REMARKS

This Amendment is in response to the Office Action dated April 19, 2007 in which claims 1-12 and 29-35 have been rejected. The rejections are traversed. Reconsideration is requested.

Claims 2-12, 29-30 and 33-36 are currently pending in the application. Claim 33 is independent.

Claim Rejections 35 U.S.C. § 112- First Paragraph - Written Description Rejection

Claims 1-12 and 29-32 are rejected under 35 U.S.C. §112 - First Paragraph, asserting that claims directed to a derivative fail to comply with the written description requirement. Applicant does not agree.

The statement that the Applicant was not in possession of any derivatives of cyclopamine is mistaken. Several derivatives of cyclopamine as defined on page 10, lines 8-26 of the specification of application were well-known publicly in prior art.

The inhibition of Hedgehog (Hh) signaling by teratogenic *Veratrum* alkaloids, e.g. cyclopamine (11-deoxojervine) and jervine, had been known (see the publications cited on page 3, lines 5-6 of specification) and for example the publication cited on page 3, line 13 of specification describes a synthetic derivative of cyclopamine that inhibits Hh signaling. Molecular structures and configuration features distinguishing the teratogenic from non-teratogenic steroidal alkaloids of *Veratrum* plants had also been identified correctly in prior art (Keeler RF, Teratology 1970;3:169-173). Total chemical synthesis of these naturally occurring steroidal alkaloids (both those that inhibit Hh signaling and not) and of various derivatives thereof had been described in prior art along with detailed physical and chemical characteristics of each (e.g. Kutney JP et al, Canadian Journal of Chemistry 1975;53:1796-1817). It is not necessary nor is it desirable to elaborate in the specification of an application on the publicly well-known matter in art. A person skilled in the art would readily distinguish a molecule that is suitable to use in the described method from a molecule that is unsuitable by reference to the clear functional definition of a "derivative of cyclopamine" in specification (page 10, lines 8-26) especially considering that particular examples of such compounds had been well known in art.

Representative specific compounds qualifying for the functional definition given on page 10, lines 8-26 of specification were common knowledge at the time of filing of present application and examples of relevant prior art scientific publications are cited in the specification as noted above. Structural features (complete molecular structure) as well as the functionality in inhibition of Hh signaling were publicly known. Requirement for teratogenicity of the rigid positioning of the piperidine ring in cyclopamine and in other teratogenic *Veratrum* alkaloids with respect to the steroid system as well as the absence of said structural feature in the non-teratogenic *Veratrum* alkaloids had been identified correctly (see Keeler RF, Teratology 1970;3:169-173, page 170, right column, first paragraph, penultimate sentence). A skilled person is well versed in the publicly available scientific-technical information. He/she has no uncertainity as to the functional characteristics of a "derivative of cyclopamine" (from the specification of application) and he/she would know not only the particular examples of a derivative described in prior art but also the structure-function relationships described therein.

In view of the foregoing explanations and facts, Applicant respectfully disagrees with the Written Description rejection. However, having stated this disagreement for the reasons of record, Applicant has amended the claims for the purpose of advancing prosecution. Applicant has cancelled claims 1 and 31-32 and amended claims 2, 3, 5 and 30 to depend from independent claim 33, making further changes as needed in some of these claims to conform to the antecedent language of claim 33. Claim 7 has also been amended to conform to the antecedent language of claim 33. Claim 29 has been made dependent on claim 2. The Written Description rejection has thus been rendered moot. Withdrawal of the rejection is respectfully requested.

Claim Rejections - 35 U.S.C. § 102 - Anticipation Rejection

Claims 1-6, 8-9, 11-12 and 29-35 have been rejected as anticipated by Beachy et al, US 6432970 (Beachy et al '970). The rejections are traversed. Beachy et al '970 does not anticipate the invention described in any of claims 1, 31 or 33. Hence, Beachy et al '970 does not anticipate the invention of any of the currently pending claims.

The factual determination of anticipation requires the disclosure of each and every element of the claimed invention in a prior art document or else the claimed invention must be inherently present in prior art. A prior art document must enable the invention for it to be anticipating and mere naming of a subject matter is insufficient for enablement, if the claimed invention cannot be made without undue experimentation. Determination of enablement must be made by taking the evidence as a whole, in particular when the teachings in a document are self-contradicting and ambiguous. In addition, when a patent is making teachings that are inoperative and an undue experimentation is required to distinguish inoperative embodiments from those that are operative, then there is a clear failure of enablement.

A. Objection to the Rejection

The disclosure in Beachy et al '970 is largely overlapping (identical word-by-word over extended passages) with that of US 6291516 (Dudek et al '516), US 6686388 (Dudek et al '388) and US 6867216 (Beachy et al '216). The anticipation rejections based on Dudek et al '516 that were made in the Office Action mailed on March 25, 2005 to the originally presented claims of this application have been withdrawn in the Office Action mailed on September 11, 2006 following the Amendment filed on March 9, 2006.

Because of the largely overlapping disclosures of all four US patents 6291516, 6432970, 6686388 and 6867216 and because the Office Action mailed on September 11, 2006 indicated that the latter three US patents were considered pertinent to the disclosures of present application although not relied upon at that time, the disclosure of Beachy et al '970, and its application in the present rejection are seen to be substantially cumulative of the previous rejections on Dudek et al '516 that have already been overcome. The Office Action does not identify a non-cumulative basis for switching to the Beachy et al '970 document to formulate the current rejection. The rejection is therefore objected to as unnecessarily duplicative and unseasonably presented.

Despite this objection, the rejection is dealt with herein on the merits. However, in an effort to avoid possible further prosecution delays involving this group of documents, the teachings of Beachy et al '970 will be analyzed here in conjunction with the teachings of other mentioned US patents. Reference will be made in addition to the international patent application published as WO 99/52534. The US 6432970 and WO 99/52534 have the same inventors, claim to the same priority data and have largely overlapping (nearly identical) disclosures.

B. Beachy et al's Teachings Taken As A Whole

Beachy et al '970, like Dudek et al '516, Dudek et al '388, Beachy et al '216 and WO 99/52534, is an omnibus disclosure pertaining to a "subject method" that comprises administration of a regulator of Hh pathway to a cell *in vitro* or *in vivo*. An unlimited number of compounds are disclosed that may act as agonist or antagonist of Hh signaling (see US 6432970, column 9, lines 4-6 and column 19, line 22 to column 29, line 32). Besides the unlimited number of compounds that are disclosed, all four of the above-mentioned US patents and WO 99/52534 disclose a similar or nearly identical long list of highly heterogenous conditions that are allegedly treatable by the "subject method" employing a "subject compound". The conditions that are taught to be treated by the "subject method" include conditions that are poorly defined or undefined ("… methods of the present include … the regulation of … lung, liver … etc"; see US 6432970, column 8, lines 38-48). Thus, except when a clarification is made (or can be inferred) as to which subject compound is to be used and what effect may be expected by the application of "subject method" in a specific condition, the disclosures are ambiguous. The ambiguity and lack of enablement is compounded by the fact that the unsupported disclosures (i.e. those that are not supported by experimental data or by reference to findings in scientific literature) are made commonly using unspecific verbs (uninformative in context) and/or are self-contradicting (see below). The teaching is also inoperative in entire categories of disclosed conditions and the attainment of a therapeutic effect by the described method is simply not possible.

For example, Beachy et al '970 (column 32, line 61 to column 33, line 9) specifically teaches that the subject method employing an antagonist of Hh signaling is to be used in treatment of traumatic injuries of nervous system, of multiple sclerosis and of related disorders. However, it is now well established that Hh signaling is required for the remyelination of neurons in demyelinating nervous system disorders, including multiple sclerosis, related demyelinating diseases and trauma to neurons. Consequently, it is the administration of an upregulator (agonist) of Hh signaling that provides therapeutic effect in these conditions and the administration of a Hh antagonist would cause rather worsening of said disorders (see e.g. the scientific publication cited on page 11, first paragraph of the *RCE With Amendment* that was filed in this application on March 9, 2006). In accord, administration of a specific antagonist of Hh signaling (cyclopamine) to rats following traumatic injury of axons caused loss of the injured neurons in a dose-dependent fashion (Akazawa C et al, The Journal of Neuroscience 2004;24:7923-7930; a copy is filed herewith).

Similarly, with regard to the effects of administration of a specific antagonist of Hh signaling on proliferation of cells in an animal, it has been well documented that different cell types respond differently and that the proliferation of some cells is stimulated while proliferation of some other cells is inhibited in the same animal (see e.g. page 11, second paragraph of the *RCE With Amendment* filed in this application on March 9, 2006 and the scientific publications cited therein). Further, whereas the claimed method in Beachy et al '970 teaches administration to "… a patient diagnosed with a hyperproliferative disorder … a purified hedgehog antagonist in a sufficient amount to reduce the activation of the hedgehog-patched pathway in a cell of the patient" (see claims 1-6; see also column 44, lines 14-24), it has been described that the amount of a specific Hh antagonist that is required for inhibition of cellular proliferation is magnitudes greater than that is sufficient for inhibition of Hh signaling (~ 100 to ~ 1000 fold greater in two examples; see Romer JT et al, Cancer Cell 2004;6:229-240; a copy is filed herewith). It is thus clear that, even in absence of ambiguity of disclosure, an undue experimentation has to be carried out by a person skilled in the art to determine which teachings are operative and which ones are inoperative in Beachy et al '970 (and in Dudek et al '516, Dudek et al '388, Beachy et al '216 and WO 99/52534).

C. Teachings on Cellular Differentiation

The unsupported teachings in Beachy et al '970 are commonly ambiguous (hence uninformative) specifically with regard to the utility of "subject method" in achieving a desired cellular differentiation outcome. Cellular differentiation is said to be "affected" or "altered" or "changed" or "controlled" or "regulated" by the subject method employing a subject compound (see e.g. column 9, lines 8-15; column 29, lines 34-38; column 30, lines 3-4; column 30, lines 14-18; column 32, lines 4-8; column 33, lines 56-61). The preponderance of such unspecific verbs emphasizes the uninformative nature of related teachings. A similar preponderance of such unspecific words is seen also in Dudek et al '516, Dudek et al '388, Beachy et al '216 and WO 99/52534 concerning the utility of subject method in obtaining a desired cellular differentiation outcome.

The disclosures concerning the utility of subject method employing a subject Hh antagonist are in addition directly self-contradicting concerning the effects on differentiation of epithelial cells. Thus, it is stated on column 41, lines 43-51 in Beachy et al '970 that a Hh antagonist can be used to induce differentiation of epithelial cells whereas it is stated on column 39, lines 10-15 that a Hh antagonist can be used to inhibit differentiation of epithelial cells. These ambiguous and self-contradicting disclosures have no utility to the skilled person. Similarly, the teaching that is cited in the Office Action that "An effective amount of … a hedgehog antagonist … brings about … a change in the rate of cell proliferation and/or state of differentiation of a cell and/or rate of survival of a cell" (Beachy et al '970, column 9, lines 8-15) may mean a change in the direction of inhibition or in the direction of induction of a differentiated state and therefore lacks utility to a person skilled in the art.

Beachy et al '970 does have teachings relating to Hh signaling effects on cellular differentiation which are based on the results of experiments described in said patent or in scientific publications cited therein. These teachings taken as a whole lead to the conclusion that the use of a Hh antagonist will inhibit cellular differentiation.

The Hh family of proteins were discovered originally as inducers of differentiation of various cell types during development. An antagonist of Hh signaling would thus ordinarily be expected to act to inhibit said differentiation events. Table 1, below, shows that the experimental findings that are described in US 6432970 commonly point to an inhibitory action of Hh antagonists on cellular differentiation.

Table 1. The Supported Teachings In The US Patent 6432970 Teach That The "Subject Method" Employing An Antagonist Of Hh Signaling Will Produce An Inhibition Of Differentiation Effect

Column and Lines	Teaching
Col. 2, lines 62-63	"… Shh … induce ventral cell fates" (references are given)
Col. 3, lines 4-12	"… Shh protein induces floorplate and motor neuron … antibody blocking suggests that Shh … is required for … induction of motor neuron fates" (references are given)
Col. 3, lines 39-42	"… filter barrier experiments suggest that Shh mediates the induction of sclerotome directly" (reference is given)

Col. 5, lines 9-29	Referring to Fig.3, describes induction of the floor plate and motor neurons by Shh and states that said inductions of differentiation are fully inhibited by sufficiently high concentrations of cyclopamine or jervine.
Col. 6, lines 22-49	Referring to Fig.6, describes induction of the floor plate and motor neurons by the Shh-N protein and states that said inductions of differentiation are "completely blocked" by sufficiently high concentrations of cyclopamine or jervine.
Col. 39, line 65 to Col. 40, line 2	"… Ihh … promote … photoreceptor differentiation" (reference is given)
Col. 40, lines 3-10	"… treatment … with … Sonic hedgehog protein results in an increase in … rod photoreceptors, amacrine cells and Muller glial cells (reference is given)
Col. 58, lines 12-19	"… cyclopamine and jervine … a complete loss of HNF3β and Isl-1 expression consistently caused by sufficiently high concentrations"
Col. 60, lines 4-17	"… Shh-N protein … induces ventral markers such as Isl-1 and HNF3β … teratogenic compounds are able to block … induction of Isl-1 and HNF3β"
Col. 62, line 66 to Col. 63, line 2	"We have … demonstrated that these compound block the induction by Shh protein of ventral cell types"

Beachy et al '970 has in addition the statement that "*in light of the … broad involvement of hedgehog proteins in the formation of … differentiated tissues in vertebrates*", the use of Hh proteins is contemplated for the generation of various vertebrate tissues in general (column 29, lines 39-45). Thus, what is shown experimentally and what is contemplated is the induction of differentiation of various vertebrate cell types by use of Hh signaling agonists and it is described that said differentiation events are inhibited by Hh signaling antagonists.

Furthermore, the exemplification in Beachy et al '970 shows that the Sonic hedgehog protein (Shh) induces differentiation of medial neural plate cells to motor neurons as well as to floor plate cells and it describes an assay for determining effects of the compounds like jervine and cyclopamine (11-deoxojervine) on said induction of differentiation (see column 57, line 67 to column 58, line 11). Using the developed assay, it is further shown that a complete inhibition of the induction of differentiation by Shh was "consistently caused by sufficiently high concentrations" of jervine or cyclopamine (see column 58, lines 12-19). Thus, Beachy et al '970 explicitly teaches that an assay has been developed for determining the Hh signaling effects on induction of differentiation and that the experiments employing said assay show that administration of sufficiently high dosages of cyclopamine or jervine causes inhibition of Hh signaling as well as a complete inhibition of differentiation. Accordingly, it is clear that the technical effect taught to be obtained in said patent by the administration of a sufficiently high dosage of an inhibitor Hh signaling is an inhibition of differentiation effect. Consequently, the induction of differentiation effect by the administration of a sufficiently high dosage of cyclopamine (or of another inhibitor of Hh/Smo signaling) that is described in the present application cannot possibly be anticipated by Beachy et al '970.

D. Teachings On Cell Survival/Apoptosis

The unsupported teachings in Beachy et al '970 about the effects of the subject method employing an antagonist of Hh signaling on apoptosis or cell survival are again ambiguous (uninformative). Thus, the teachings cited in the Office Action speak of "changing" or "modulating" survival of a cell by the subject method employing a subject Hh antagonist (column 9, lines 8-14; column 29, lines 34-38). Since changing or modulating cell survival may mean increasing or decreasing it, the quoted teachings are uninformative and have no utility to a person skilled in the art.

Referring to the requirement of cellular proliferation for causation of cell death by cyctotoxic chemotherapeutics and by radiation therapy in conventional cancer therapies, Beachy et al '970 teaches specifically that the subject method using a Hh antagonist can be used to inhibit cellular proliferation and thereby to prevent apoptosis or programmed cell death "… *because a hedgehog antagonist will often be cytostatic to epithelial cells rather than cytotoxic*" (see column 40, line 57 to column 41, line 1). In contrast to this general teaching about the expected effect of a hedgehog antagonist, an unsupported teaching in the same document (column 33, lines 24-26) states that Hh antagonists can be used to cause transformed cells to become apoptotic. The Office Action makes a reference to this latter statement. A person skilled in the art, when presented with unsupported and contradictory teachings, would take into account the actual experimental data and evidence as a whole. Beachy et al '970 has two descriptions of actual experiments where a specific inhibitor of Hh signaling was administered onto tumor cells. In neither instance a description is made of the causation of tumor cell death by said administration. US 6432970 refers, on column 35, lines 46-59, to experiments where human lung cancer cells were treated with a specific inhibitor of Hh signaling. Despite causation of a marked inhibition of tumor cell proliferation by said treatment and despite the microscopic examination of proliferation-inhibited cells, no description of tumor cell death is found (see the cited Fujita E et al, Biochemical and Biophysical Research Communications 1997;238:658-664 article; a copy is filed herewith). In the other experiments described in Beachy et al '970 where an inhibitor of Hh signaling was administered onto tumor cells, liver cancer cells were treated with cyclopamine or with jervine (see column 68, lines 14-24).

Other prior art documents and patents, including Dudek et al '388 and Beachy et al '216 also describe experiments where a specific inhibitor of Hh signaling was administered onto tumor cells, including the transformed cells exhibiting constitutive Hh signaling due to a lack of a functional *patched* allele. Beachy et al '216 and the international application WO 99/52534 have the same inventors as Beachy et al '970 and largely similar or identical disclosures. They describe experiments where the subject method employing cyclopamine was applied onto transformed cells exhibiting constitutively activated Hh signaling and teach explicitly that the use of subject method causing a marked inhibition of Hh signaling in said transformed cells did not cause cell death (see US 6867216, column 76, lines 24-33; WO 99/52534, page 102, lines 23-29).

Dudek et al '388 describes experiments where brain tumor cells exhibiting constitutive activation of Hh signaling were treated with a specific inhibitor of Hh signaling (jervine or cyclopamine) *in vitro* as well as *in vivo*. Whereas the subject method employing a specific inhibitor of Hh signaling is described in this patent to produce strong inhibition of tumor cell proliferation, no description of a causation of tumor cell death is made even though the proliferation-inhibited tumor cells were examined microscopically (see US 6686388, column 63, line 56 to column 64, line 52). As explained in the 1/10/2007 dated Amendment filed in this application in analysis of the similar disclosures of Dudek et al '516, such microscopic examinations would allow detection of apoptosis. Furthermore, again as explained in said

Amendment, *the dosage of cyclopamine that is described in US patents 6686388 and 6291516 for producing inhibition of tumor growth in vivo is magnitudes lower than the dosages described later in independent scientific investigations to produce apoptosis of the same tumor cells and of similar other tumor cells in vivo.* Still further, as documented in the 1/10/2007 dated Amendment, the attached Declaration and the scientific publications filed therewith, *a minimal dosage of cyclopamine that is sufficient to produce inhibition of Hh signaling and inhibition of tumor cell proliferation is clearly insufficient to produce tumor cell apoptosis.*

In conclusion, it is clear that neither Beachy et al '970 nor the other prior art documents anticipate the presently described method that employs a specific inhibitor of Hh/Smo signaling to produce highly efficient and selective induction of apoptosis of tumor cells *in vivo*. It is further clear that the presently described technical effects were not inherently present in a prior art method using a specific inhibitor of Hh signaling.

A scientific publication (Tas S, Melanoma Research 2006;16:S99) and an as yet unpublished manuscript by the first-named inventor in this application are filed herewith and demonstrate that the unprecedented efficiency and selectivity *in vivo* of the inductions of tumor cell differentiation and apoptosis that are described in this application are not irreproducible chance events. Rather, the causations of said technical effects are based on the solution of a fundamental difference between the normal and tumorigenic cells displaying stem cell features and the described method of treatment is a rational intervention designed to take advantage of said fundamental difference.

E. Comments in The Office Action Concerning Anticipation

Citing the disclosures in Beachy et al '970 that steroidal alkaloids including cyclopamine can be used to "inhibit the bioactivity of hedgehog proteins" (column 7, lines 50-54) and that a therapeutically-effective amount of a subject compound is defined as the amount "which is effective for producing some desired therapeutic effect by inhibiting a hedgehog signaling pathway in at least a subpopulation of cells in an animal and thereby blocking the biological consequences of that pathway in the treated cells" (column 45, lines 9-17), the Office Action states that US 6432970 anticipates inducing differentiation and apoptosis in a cell if the "biological consequence" of hedgehog signaling is inhibition of differentiation and apoptosis.

Speculation as to a possible biological consequence for a hypothetical cell has no enablement and has no place in anticipation analysis. A disclosure that allows for multiple therapeutic effects of a compound, and in that context teaches to use a therapeutically effective amount of the compound, does not thereby anticipate administering an amount which produces any specific one of those effects. This is because each one of those effects is not necessarily produced by an effective amount within that disclosure. See *Amgen Inc. v Hoechst Marion Roussel Inc, 79 USPQ2d 1705 (Fed Cir 2006).* Thus, US 6432970 is only relevant for anticipation purposes for the specific technical effects taught to be obtained in treatment of specific cells, or those which necessarily occur in the described treatment.

A specific technical effect of the administration of an effective amount of an inhibitor of Hh signaling onto target cells that is taught in Beachy et al '970 to be consistently obtained is the inhibition of cellular differentiation that is normally induced by a Hh protein (see above, under the heading *C. Teachings On Cellular Differentiation*). The Office Action also points out to the teaching of Beachy et al '970 that Hh proteins specify cell identity in diverse systems (column 7, lines 8-12). Said *teachings about the bioactivity of Hh proteins* make it impossible to declare that Beachy et al '970 anticipates induction of differentiation of the target tumor cells by the administration of an effective amount of a Hh antagonist.

Not all cell types in an animal respond similarly to the administration of a Hh antagonist and the specification of present application shows that inductions of differentiation and apoptosis are not consequences in all cells treated with cyclopamine. Normal, non-tumorigenic cells that are exposed to the same amount of cyclopamine as the tumor cells are not induced to undergo differentiation and apoptosis. Furthermore, it has been shown that a minimal dosage of a specific antagonist of Hh signaling that is sufficient to inhibit Hh signaling in the target tumor cells is insufficient to exert an inhibition of proliferation effect (see Romer JT et al, Cancer Cell 2004;6:229-240). Still further, a minimal dosage of a specific antagonist of Hh signaling that is sufficient to inhibit proliferation of the target tumor cells is insufficient to exert an induction of apoptosis effect (see the Tas Declaration and attached scientific publications filed with the 1/10/2007 dated Amendment in this application). Inhibitions of Hh signaling and of proliferation are "biological consequences" that do not meet the recitation of claim 33. Consequently, even if for the sake of argument the Beachy et al '970 is taken as disclosing treatment of tumor cells in an effective amount for producing a "biological consequence," such would not be sufficient to constitute an anticipation of claim 33 as a matter of law.

The Office Action also states on page 8 that "With respect to an effective amount of a compound, which reasonably correlates with the instantly claimed sufficient quantity, Beachy et al teach that an effective amount … brings about … a change in the rate of cell proliferation and/or rate of survival of a cell … The reference thus reasonably anticipates inducing differentiation and apoptosis … by administering a hedgehog antagonist". These quoted teachings mean that Beachy et al considers that any dosage that produces any effect is an effective amount.

Since "a change in the rate of cell proliferation and/or the state of differentiation of a cell and/or rate of survival of a cell" literally encompasses an increase in cell survival, ***as a matter of law***, Beachy et al do not "reasonably anticipate[s] inducing differentiation and apoptosis (rate of survival) by administering a hedgehog antagonist." Inducing differentiation is merely one way in which a differentiation state may be altered. Inducing apoptosis is merely one way in which survival rate may be changed. Therefore the Office Action clearly errs in making this anticipation assertion. The Office Action has impermissibly relied upon possibilities, rather than specific or "necessarily present" consequences in the anticipation analysis.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California,* 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). The identical invention must be shown in as complete detail as is contained in the ... claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). "[T]o constitute an anticipation, all material elements recited in a claim must be found in one unit of prior art." *In re Marshall* , 578 F.2d 301, 304, 198 USPQ 344, 346 (CCPA 1978); *Ex parte Gould* 6 USPQ2d 1680 (BdPatApp&Int 1987). *See also,* MPEP 2131. Reliance on inherency in an anticipation analysis requires evidence that the missing descriptive matter is "necessarily present" in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. MPEP §2131.01 (III); *Continental Can Co. USA v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991). ***Inherency may not be established by probabilities or possibilities.*** The mere fact that a certain thing may result from a given set of circumstances is not sufficient. *Continental Can*, 948 F.2d at 1269, 20 USPQ.2d at 1749; *In re*

Robertson, 49 USPQ2d 1949, 1951 (Fed. Cir. 1999).

Similarly, the teaching quoted from Beachy et al '970 at column 44 (lines 26-29) speaks of a dosage range of one million fold difference between the lowest and highest doses and follows immediately the teaching on the same column that the lowest dose effective to produce a therapeutic effect described therein is to be used (see lines 14-24). As already noted, the lowest dose effective to produce an inhibition of Hh signaling in the target tumor cells is insufficient to produce inhibition of proliferation (magnitudes difference) and the lowest dose sufficient to produce inhibition of proliferation is insufficient to produce induction of apoptosis (another magnitudes difference). It is thus clear that *the presently described sufficient amount is necessarily different from the effective amount taught in prior art and it produces results unexpected in prior art.* Consequently, Beachy et al '970 cannot be declared anticipating.

F. Conclusion on Anticipation

Consideration of the whole of the disclosure of Beachy et al '970 as well as of the entire prior art shows clearly that the presently described and claimed invention was unanticipated in prior art and was not inherently present in prior art. Withdrawal of the anticipation rejection on Beachy et al '970 is respectfully requested.

Claim Rejections - 35 USC §103

Claims 7 and 10 are rejected under 35 USC § 103 as being rendered obvious by Beachy et al '970. Applicant does not agree.

Claims 7 and 10 are both dependent on claim 5, which in turn is dependent on claim 33. Beachy et al '970 does not meet the recitations of claim 33 for the reasons already given. Furthermore, objective evidence of the unobviousness of the invention has been presented in the 1/10/2007 dated Amendment filed in this application. Claims 7 and 10 depend from a claim that defines a novel and unobvious invention. They are thus allowable. Withdrawal of the obviousness rejection is respectfully requested.

Conclusion

In view of the foregoing amendments and remarks, the outstanding rejections are seen to have been overcome. The presently claimed invention is novel and unobvious over the US patent 6432970 and entire prior art. It has brought a highly desirable solution to long-standing problems in tumor treatment. Withdrawal of the outstanding rejections and allowance of the application is respectfully requested.

Respectfully submitted,
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Development/Plasticity/Repair

The Upregulated Expression of Sonic Hedgehog in Motor Neurons after Rat Facial Nerve Axotomy

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Nerve injury leads to the induction of a large number of genes to repair the damage and to restore synaptic transmission. We have attempted to identify molecules whose mRNA expression is altered in response to facial nerve axotomy. Here we report that facial nerve axotomy upregulates Sonic hedgehog (Shh) and its receptor Smoothened (Smo) in facial motor neurons of adult rats, whereas facial nerve axotomy does not upregulate mRNA of Shh or Smo in neonatal rats. We tested whether overexpression of Shh in facial motor neurons of axotomized neonatal rats may promote neuronal survival. Adenovirus-mediated overexpression of Shh, but not that of β-galactosidase, transiently rescues axotomy-induced neuronal cell death for 3–5 d after axotomy. Finally, the pharmacological inhibitor of Shh signaling, cyclopamine, induces motor neuron death in adult rats after axotomy. These results suggest that Shh plays a regulatory role in nerve injury.

Key words: Shh; neuronal death; axotomy; facial nerve; motor neuron; adenovirus

Introduction

The neuronal responses after peripheral nerve injuries have been intensively characterized and are certainly one of the best characterized models of neuronal regeneration. Facial nerve axotomy of rats, with its extensive abilities to regenerate in adults, provides us with a useful model in which to study the molecular mechanisms of nerve regeneration. It is well known that facial nerve axotomy in neonatal rats induces motor neuron death of the facial nucleus, whereas most of the neurons survive in adult rats (Graeber et al., 1998). The lack of regenerative properties of neonatal rat neurons is partly attributable to insufficient trophic supports from their target (Snider, 1989; Sendtner et al., 1992; Schnell et al., 1994; Yan et al., 1995; Fournier and McKerracher, 1997), but the molecular mechanisms of the axotomy-induced neuronal death in neonatal rats remain to be fully elucidated. To investigate the mechanisms that are responsible for promotion of motor neuron survival, we searched molecules whose mRNA expression is altered after nerve injury. We found that Sonic hedgehog (Shh) expression was significantly upregulated in the cell bodies of motor neurons after facial nerve axotomy.

Shh has been intensively characterized in the developmental stages of various organs, including motor neuron differentiation (Richardson et al., 1997; Pfaff and Kintner, 1998; Briscoe et al., 1999; Souza et al., 2001), limb development (Te Welscher et al., 2002), hair follicle formation, and odontogenesis (St-Jacques et al., 1998). In the developing neural tube, the secretion of Shh from the notochord and floor plate organizes the pattern of ventral neurogenesis along the entire length of the CNS. Shh also plays an important role in the phenotypic specification of ventral neurons. For example, Shh induces the differentiation of motor neurons in the spinal cord and dopaminergic neurons in the midbrain (Tanabe et al., 1995; Pfaff and Kintner, 1998; Briscoe et al., 1999). Although the expression of Shh persists in the adult brain beyond this induction period, little is known about its functional role in adults. A goal of this study is to characterize Shh expression in matured motor neurons after nerve injuries. We performed adenoviral gene transfer of chick Shh to overexpress Shh in the axotomized motor neurons of neonatal rats as a gain-of-function approach. Finally, we analyzed the effect of pharmacological inhibition of Shh signaling in the axotomized motor neurons of adult rats as a loss-of-function approach.

Materials and Methods

Animals. Adult Wistar rats (200–250 gm) were housed in the animal facility of National Institute of Neuroscience, National Center of Neurology and Psychiatry. The first 24 hr after birth is postnatal day 0 (P0). All animal experiments and care protocols were performed under the approval of the ethical committee on laboratory animals of the institute.

Recombinant adenovirus vectors and Shh antagonist application. The construction and characterization of the adenovirus (AdV) carrying the gene for chick Shh or β-galactosidase (lacZ) was described previously (Ohsaki et al., 2002). The AdV was concentrated by cesium chloride density gradient centrifuge. The concentrated AdV was diluted with PBS. Cyclopamine (Toronto Research Chemicals, North York, Ontario,

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Canada) or tomatidine (Sigma, St. Louis, MO) was dissolved at a concentration of 1.0 or 5.0 μg/ml in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin (HBC) (Sigma) in PBS (Berman et al., 2002; Lai et al., 2003).

Facial nerve transection. Under ether anesthesia, the facial nerve was transected at the stylomastoid foramen (Graeber et al., 1988). In all experiments, the unoperated contralateral sides serve as a control. For the AdV type V vector, cyclopamine or tomatidine application involved a 1.0 mm^3 Gelfoam (Amersham Biosciences Japan, Tokyo, Japan) soaked with a 1.0×10^{10} multiplicity of infection of AdV, 3 μl of cyclopamine (1.0 or 5.0 μg/ml), or 3 μl of tomatidine implanted immediately at the nerve cut site. After the indicated intervals (6, 12, and 24 hr and 3, 5, 7, and 14 d), rats were killed by cervical decapitation under deep anesthesia by ether. The brains were quickly removed without perfusion and frozen at −70°C until use. Whole series of coronal sections (14-μm-thick cryosections) were taken through the full extent of bilateral facial nuclei. The number of survived motor neurons was determined and counted by a blinded observer as described previously (Baumgartner and Shine, 1997). Five independent experiments were performed, and the total number of survived motor neurons was statistically analyzed (Student's *t* test).

Subtraction cDNA library screening. Five series of pooled facial nuclei were subjected to total RNA isolation by the phenol-guanidine isothiocyanate method. Subtractions between axotomized nuclei and contralateral nuclei were performed by using the SMART subtraction kit (BD Biosciences Clontech, Palo Alto, CA). Three hundred clones were randomly sequenced to identify the insert cDNA.

In situ hybridization and immunohistochemistry. The coding region of rat Shh cDNA (accession number NM_017221) was amplified by PCR using paired primers (5′-ATGCTGCTG-CTGCTGGCCAGA-3′ and 5′-TCAGCTGGA-CTTGACTGCCAT-3′). The cDNA fragments for rat Patched1 (Ptc1) (accession number AF079162) and rat Smoothened (Smo) (accession number U84402) were amplified by the paired primers 5′-ATGCTGAATAAAGCCGAAGT-3′ and 5′-CACCACGCTGA-CACAGGGC-3′ for Ptc1; and 5′-CTTCCGGGACTATGTGCCAT-3′ and 5′-AGAAGTCCGAGTCTGCATCC-3′ for Smo. The amplified fragment was subcloned into a pGEM-T easy vector (Promega, Madison, WI) and subsequently sequenced. Antisense and sense riboprobes were synthesized by transcription using either T7 or SP6 RNA polymerase (Roche Diagnostics, Mannheim, Germany) in the presence of digoxigenin-UTP (Roche Diagnostics) for 30 min at 37°C according to the manufacturer's protocol. The digoxigenin-labeled RNA probe was column-purified and reconstituted in distilled water. The 16-μm-thick coronal sections containing bilateral facial nuclei were fixed in 4% paraformaldehyde in 0.1 M PBS for 20 min and washed in PBS, and sections were hybridized overnight at 65°C in a mixture (50% formamide, 10 mM PBS, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10% dextran sulfate, 1× Denhardt's reagent, 0.2% sodium lauryl sarcosine, 500 μg/ml tRNA, and 200 μg/ml sonicated salmon sperm DNA) containing labeled probe (0.5 μg/ml in hybridization mixture). After hybridization, sections were washed in 10 mM dithiothreitol in 5× SSC at 55°C. After washing, alkaline phosphatase-conjugated anti-digoxigenin antibody staining was performed following a chromogenic reaction containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. After capturing the images, sections were counterstained with Nissl, dehydrated in a graded series of ethanol, and coverslipped with DePeX (BDH Chemicals, Poole, UK).

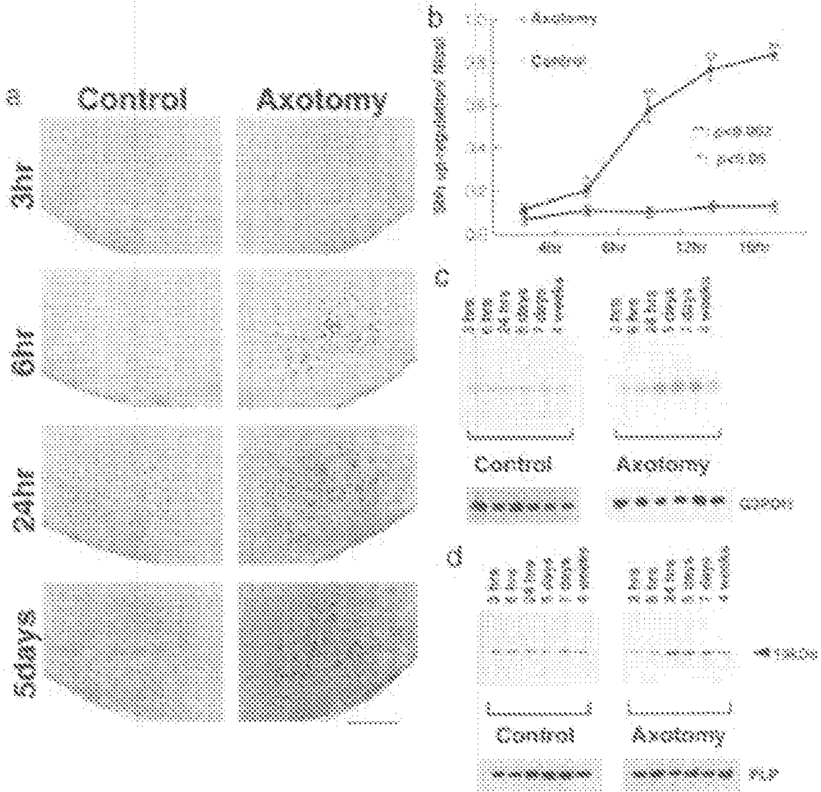

Figure 1. *a*, Upregulated expression of Shh after axotomy in adult rats. In situ hybridization of Shh antisense riboprobes in a coronal section at 3, 6, and 24 hr and 5 d after the axotomy. The relatively large size of cells corresponding to motor neurons in facial nucleus shows the expression of Shh mRNA. Compared with the control side (left), the signal intensity was several-fold higher in the axotomized facial nucleus (right). Scale bar, 500 μm. *b*, After capturing the in situ hybridization image, the section was stained with Nissl to calculate the ratio of Shh-upregulated cells. The number of motor neurons that display upregulation of Shh increased to 83 ± 3.4% at 18 hr after the operation. *c*, *d*, Quantitative analyses of Shh expression after axotomy by Northern blot (*c*) and Western blot (*d*) analyses. Each lane contains 20 μg of total RNA (*c*) and 10 μg of total protein (*d*) purified from pooled facial nuclei. Expression of Shh mRNA and 19 kDa polypeptide increased at 24 hr after axotomy and was sustained up to 4 weeks. The control blots using radiolabeled G3PDH for Northern blot and anti-PLP antibody for Western blot revealed that relatively equal amounts of RNA and protein were loaded in each lane.

Double fluorescent-labeling analyses were performed to identify the cellular expression of adenovirus-mediated Shh using mouse monoclonal anti-Shh antibody (5E1; 1:500 dilution; Developmental Studies Hybridoma Bank) in combination with polyclonal antibodies against p75NTR (neurotrophin receptor) (rabbit anti-human p75; 1:1000 dilution; Promega), GAP-43 (growth-associated protein-43) (N-19; goat anti-human GAP-43; 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), Iba1 (rabbit anti-Iba1; 1:1000 dilution) (Imai et al., 1996), and GFAP (rabbit anti-GFAP; 1:500 dilution; Dako, Glostrup, Denmark). Cryosections fixed with 4% paraformaldehyde, 0.3% Triton X-100, and PBS were incubated with primary antibodies overnight at 4°C and washed and incubated with the secondary antibodies. Fluorescent images were captured by using a Leica confocal microscope (TSC-SP2; Leica Microsystems, Wetzlar, Germany).

Northern and Western blotting. Rat facial motor nuclei (five pooled animals) were homogenized in 500 μl of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 10% glycerin, 1% Triton X-100, 0.4 mM Na_3VO_4, 10 μg/ml aprotinine, 10 μg/ml leupeptine, and 1 mM phenylmethylsulfonyl fluoride). Lysates were then centrifuged at 12,000 × *g* for 20 min at 4°C. The protein concentration was quantified using a modification of the Bradford assay (Bio-Rad, Munich, Germany). Total cell lysate (10 μg) was electrophoresed through 7.5% SDS polyacrylamide gels and electroblotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The blots were incubated overnight at 4°C with rabbit anti-Shh (1:2000; a generous gift from A. P. McMahon, Harvard University, Cambridge, MA) or anti-goat anti-myelin proteolipid protein (PLP) (Santa Cruz Biotechnology) in 10 mM Tris HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20 containing 1% BSA.

RNase protection assay. For detection of Smoothened and Patched mRNA, equal amounts of total RNA (30 μg) were subjected to RNase protection assay as described previously (Funakoshi et al., 1993). Radiolabeled antisense riboprobes were generated in the presence of [γ-^{32}P]CTP using SP6 or T7 RNA polymerase. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used for subsequent standardization. After the RNase digestion, protected fragments were separated in urea-denatured 4% polyacrylamide gels and exposed to x-ray films with intensifying screens at −70°C.

Results

Identification of Shh as an upregulated molecule after facial nerve axotomy

We sequenced 300 clones from the subtraction cDNA library between axotomized and contralateral facial nuclei at 24 hr after axotomy. By searching the BLAST database, 182 clones were identical to transcripts from known genes, and 118 clones were unknown (data not shown). Some of the known genes, such as galectin-1 (Akazawa et al., 2004), GFRα-1 (glial cell line-derived neurotrophic factor family receptor α1) (Tsujino et al., 1999), GLT-1 (glutamate transporter-1) (Lopez-Redondo et al., 2000), p75NTR (Gschwendtner et al., 2003), and GAP-43 (Tetzlaff et al., 1991), were already described as upregulated after axotomy of facial or hypoglossal nerves. We found four transcripts of Shh and one transcript of Smo as upregulated molecules.

Upregulation of Shh in the facial motor neurons of adult rats

In situ hybridization was performed to characterize the cellular expression of Shh after facial nerve axotomy. The motor neurons of adult facial nucleus express Shh mRNA at a low level (Fig. 1a, Control). On the side of axotomy, facial motor neurons started to show Shh transcripts at 6 hr after the operation. The expression of Shh reached its peak at 24 hr and continued at high levels until 4 weeks after the operation. To quantify the population of Shh-upregulated motor neurons in the axotomized facial nucleus, the sections were counterstained by Nissl to identify motor neurons. The number of neurons that upregulated Shh transcripts and the number of Nissl-positive cells were counted in the same sections, and the ratio was shown (Fig. 1b). Almost 80 ± 1.4% of Nissl-positive neurons upregulated Shh at 18 hr after the axotomy, whereas in the control side, <20% of Nissl-positive neurons express Shh. To semiquantify the Shh increase, we performed Northern blot analysis for the total RNA purified from pooled facial nuclei from five independent experiments (Fig. 1c). Transcripts of Shh were present at a low level at 3 hr after axotomy. The initial increase was detected at 6 hr and reached its peak at ∼24 hr, followed by a subsequent decline. We next tested the protein expression of Shh by Western blot analysis. The 19 kDa single band was detected, and the expression increased from 24 hr after axotomy and was sustained for up to 1 month (Fig. 1d). From these experiments, facial nerve axotomy induces the expression of Shh transcripts and proteins in motor neurons of adult rats.

Upregulation of Smo after axotomy

The Shh receptor consists of at least two components, Ptc1 for ligand binding and Smo for downstream signaling (Ho and Scott, 2002). We next tested whether axotomy of adult rats alters the expression of the Shh receptor. We performed RNase protection analysis to detect the transcripts of Smo, Ptc1, and G3PDH, respectively (Fig. 2a). Radiolabeled antisense riboprobes corresponding to the 384 bp of Smo, 443 bp of Ptc1, and 410 bp of G3PDH yielded 302, 300, and 302 bp of protected band after RNase digestion, respectively. The expression of Smo mRNA was upregulated at 24 hr after axotomy, whereas no change in Ptc1 and G3PDH expression was observed between axotomized and control facial nuclei. The intensity of protected bands in G3PDH showed that relatively equal amounts of RNA were used in the experiments. To further investigate the cellular expression of Smo, in situ hybridization histochemistry was performed (Fig. 2b). The Smo mRNA was detected in the facial motor neurons of the control side, albeit at a low level. Facial nerve axotomy of adult rats induced upregulated expression of Smo mRNA in the motor neurons at 24 hr after axotomy. No upregulated expression of Ptc1 mRNA was detected by *in situ* hybridization (data not shown).

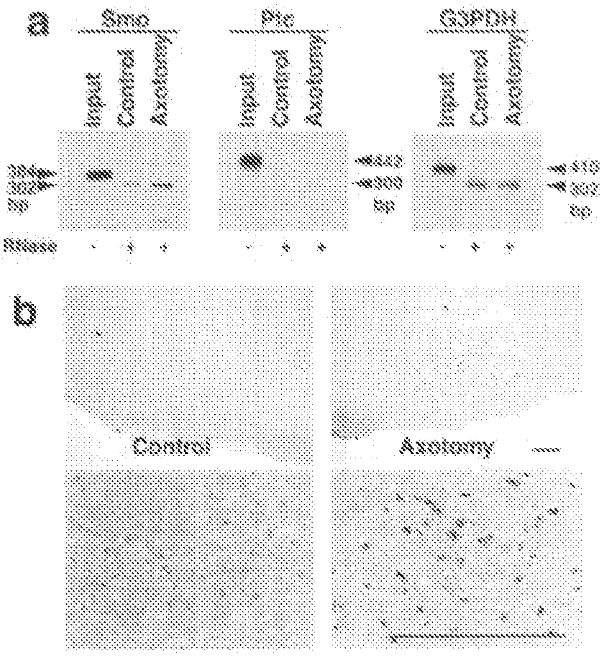

Figure 2. Expression of the Shh receptor Smo and Ptc in the facial nucleus of control and axotomized adult rats at 24 hr after axotomy. a, The total RNA (30 μg) purified from pooled facial nuclei was subjected to RNase protection analysis. For the expression of Smo, a 384 bp antisense riboprobe was hybridized, and a 302 bp band was protected after RNase digestion. For the expression of Ptc1, a 443 bp antisense riboprobe yielded a 300 bp protected band after RNase digestion. Control experiments using a G3PDH antisense riboprobe showed that equal amounts of total RNA were used in these experiments. b, In situ hybridization histochemistry showing the cellular localization of Smo transcripts in facial nucleus. The axotomy of adult rat facial nerve induced upregulated expression of Smo mRNA in the cell bodies of motor neurons. Scale bar, 500 μm.

No upregulation of Shh and Smo in neonatal rats

It is well characterized that neonatal facial nerve axotomy induces motor neuron cell death ∼5 d after axotomy. We next tested whether Shh expression was upregulated in neonates after axotomy. Although the basal level of Shh expression was detected in cell bodies of motor neurons at P2, that is, 24 hr after axotomy (Fig. 3a, Control), the facial nerve axotomy did not induce Shh expression (Fig. 3a, Axotomy). To further investigate the time course and semiquantification of molecules after axotomy, Northern and Western blot analyses were performed by using total RNA and total protein lysates purified from facial nuclei collected from five independent experiments. No upregulation of Shh transcripts and polypeptides was detected after axotomy of neonatal rats (Fig. 3b,c). The Shh expression was not detected 5 d after axotomy in the axotomized facial nuclei. This is because of the axotomy-induced neuronal death in neonates. We next tested whether axotomy of neonatal rats alters the expression level of Smo by RNase protection analysis. The protected bands were clearly detected for both Smo and Ptc1, although there was no change of expression between control and axotomized facial nuclei (Fig. 3d). Neonatal rats lack the upregulation of both Shh and Smo after axotomy.

Motor neuron survival promoted by Shh–adenovirus in neonatal rats

To test the possibility that the upregulated expression of Shh may relate to the survival of axotomized motor neurons, we implanted a Gelfoam (1.0 mm^3) soaked with a 1 × 10^{10} multiplicity of infection of AdV vector containing chick Shh (AdV–Shh) (Ohsaki et al., 2002) or β-galactosidase (AdV–lacZ) at the proximal stump of facial nerve immediately after the bilateral axotomy. First, to test the efficacy of gene transfer by AdV, we compared the lacZ staining after the operation of neonatal and adult rats. Operations were performed at P1 and adult (200–250 gm), and Gelfoams soaked with AdV–lacZ were implanted. We found that AdV-mediated gene transfer is more effective in neonatal rats than in adult rats (see Fig. 6c). Thus, we used neonatal rats for the exogenous overexpression of Shh. We applied the AdV–Shh to the right side of the axotomized nerve stump and the AdV–lacZ to the left side. Pups were treated for 4 d and then killed for sectioning. The sections were double-stained with anti-Shh rabbit polyclonal antibody and anti-lacZ monoclonal antibody and visualized with Alexa-Fluor 488- and Alexa-Fluor 594-conjugated secondary antibodies, respectively. After capturing the confocal images, the sections were stained with Nissl to obtain neuronal nuclear staining. There is a significant increase of Nissl staining-positive cells in the AdV–Shh-infected side (Fig. 4b) compared with the AdV–lacZ-infected side (Fig. 4a). Antibody staining showed the overexpressed Shh was only detected in the AdV–Shh-infected side (Fig. 4d). When we compared the Nissl staining with anti-Shh staining, Shh-transferred motor neurons formed a cluster-like staining. Western blotting analysis confirmed the overexpression of chick Shh only in the AdV–Shh-infected side (data not shown).

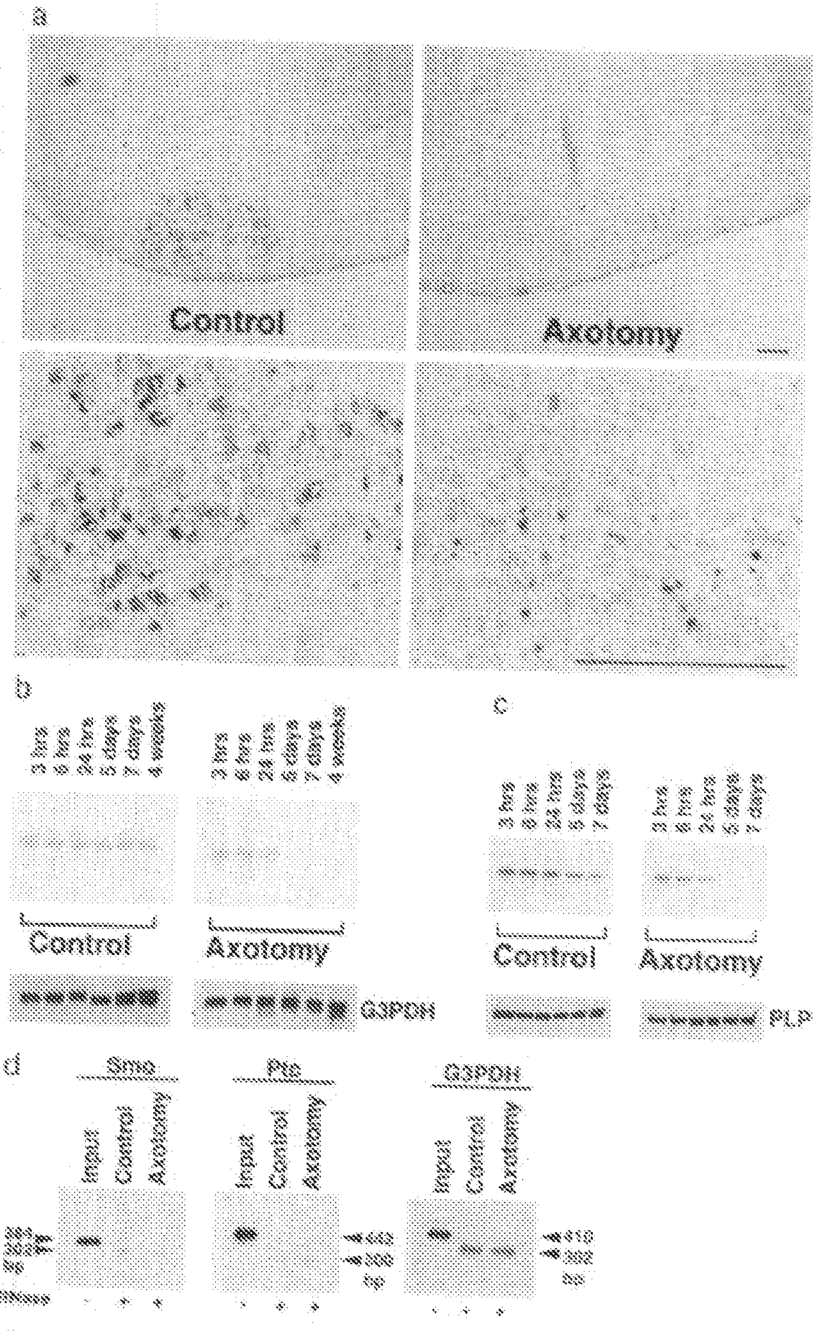

Figure 3. a, In situ hybridization histochemistry of Shh in neonatal rats at 24 h after axotomy. The control side of facial motor neurons expresses Shh mRNA at a low level, whereas axotomy of facial nerves rather suppresses the Shh expression. Scale bar, 100 μm. b, c, Quantitative analyses of Shh expression after axotomy by Northern blot (b) and Western blot (c) analyses. Each lane contains 30 μg of total RNA (b) and 10 μg of total protein lysates (c) purified from pooled facial nuclei corresponding to representative time course. No expression of Shh mRNA and protein was detected in the facial nuclei after 5 d in axotomized neonatal rats because the axotomy of neonatal rats induces motor neuron death. Control blots were performed using radiolabeled G3PDH or anti-PLP antibody. d, The RNase protection analysis revealed that axotomy of neonatal rat facial nerves did not induce the expression of Smo and Ptc1 mRNAs.

To identify the cell types infected with AdV–Shh, fluorescent double staining was performed in neonatal rats. The immunoreactivities of anti-Shh monoclonal antibody were visualized with the secondary antibody conjugated with Alexa-Fluor 488 (Fig. 5, green). Rabbit polyclonal antibodies against p75NTR or GAP-43

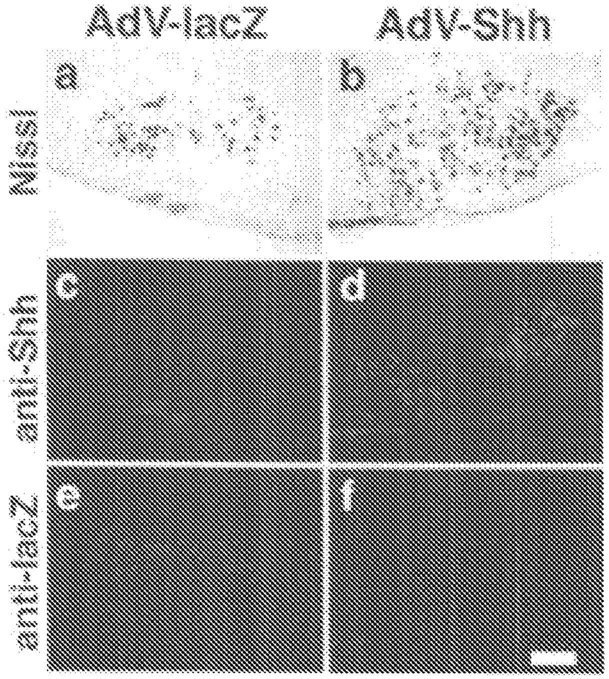

Figure 4. We tested the effect of Shh overexpression in neonatal rats by the implantation of Gelfoam containing AdV–lacZ in the left operation side and Gelfoam containing AdV–Shh in the right operation side. At 3 d after the operation, the cryosections were stained with rabbit polyclonal anti-Shh (c, d) and mouse monoclonal anti-lacZ (e, f) antibodies visualized with Alexa Fluor 488-conjugated (green) and Alexa Fluor 594-conjugated (red) secondary antibodies. After capturing the confocal images, sections were stained with Nissl to visualize the neuronal nuclear staining (a, b). Five independent experiments were performed, and a representative result is shown. Scale bar, 100 μm.

were used to identify the motor neurons of facial nucleus. For non-neuronal marker stainings, rabbit polyclonal antibodies against Iba1 or GFAP were used. The immunoreactivities of neuronal or non-neuronal markers were visualized by the secondary antibody conjugated with Alexa Fluor 594 (Fig. 5, red). The expression of Shh (Fig. 5, arrows), which was only detected in the lesioned facial nuclei, overlapped well with the immunoreactivities of p75NTR or GAP-43. Meanwhile, non-neuronal markers, such as Iba1 that localizes in the cell bodies of microglia–macrophage (Fig. 5, m) and GFAP that localizes in astrocytes (Fig. 5, q), were barely detected in the Shh-positive cells.

Time course of survival-promoting effect of Shh overexpression in neonatal rats

Given the potent *in vivo* action of AdV–Shh on axotomized motor neurons of neonates, we next looked for the time course of this effect (Fig. 6B). At 1 d after the operation, lesioned neonates that were treated with AdV–lacZ retained only $0.37 \pm 0.07 \times 10^3$ cells of their facial motor neurons. In contrast, the lesioned facial nuclei that received AdV–Shh showed $0.88 \pm 0.07 \times 10^3$ cells stained with Nissl, undistinguishable from the nonlesioned side. The difference in neuronal survivals between the AdV–Shh and AdV–lacZ was statistically significant ($n = 5$; $t = 21.9$; $p < 0.001$). However, this effect was only retained for 1 week. At 2 weeks after the operation, the motor neurons that received AdV–Shh finally shrank, and the cellular organization of the facial nucleus was no longer observed. Thus, AdV–Shh can prevent death of axotomized neonatal motor neurons, although this is transient and insufficient to attenuate the full survival. To exclude the toxicity of AdV treatment, we compared the number of the Nissl-positive neurons between the AdV–lacZ-infected facial nuclei and PBS-applied facial nuclei. There is no significant difference in the number of survived cells between AdV-infected neurons and PBS-applied neurons (Fig. 6C). This means that there is no toxic effect of AdV, and the survival of our current result may be solely attributable to the overexpression of Shh.

Inhibition of Shh signaling in adult rat axotomy

To examine whether the inhibition of Shh signaling would reduce the regeneration of axotomized motor neurons in adult rats, the Gelfoam was soaked with cyclopamine, a specific inhibitor of Shh signaling shown to act by direct binding to the heptahelical bundle of Smo (Chen et al., 2002b), tomatidine (Berman et al., 2002), an inactive analog of cyclopamine, or with the vehicle HBC and implanted at the axotomized region. HBC has been extensively used to increase the solubility of hydrophobic compounds for delivery to the brain with no observed side effects (Lai et al., 2003). At 7 d after the implantation of cyclopamine, tomatidine, or vehicle alone, sections covering bilateral facial nuclei were prepared, and the total number of Nissl-positive motor neurons was counted. Five independent rats were operated on and subjected to the statistical analyses (Student's *t* test). The implanted cyclopamine reduced the number of Nissl-positive neurons by ~60% in a dose-dependent manner compared with the control vehicle side (Fig. 7). Meanwhile, the implanted tomatidine did not influence the number of Nissl-positive neurons.

Discussion

Nerve injuries induce a variety of molecular responses such as neurotrophic factors, cytokines, growth factors, and extracellular molecules that may be involved to some extent in the regeneration of injured neurons (Skene, 1989; Doster et al., 1991; Sendtner et al., 1992; Schnell et al., 1994; Yan et al., 1995; Fournier and McKerracher, 1997). In the course of searching the molecules whose expression is altered in adult rat motor neurons of facial nucleus after axotomy, we found that Shh expression was upregulated from 24 hr after axotomy and declined at 4 weeks. The physiological roles of Shh have been intensively characterized in vertebrate development. It specifies the identity of different cell types in the ventral neural tube in early development (Pfaff and Kintner, 1998; Briscoe et al., 1999; Oppenheim et al., 1999). Although Shh is strong in inducing motor neurons from progenitor cells, there have been few reports characterizing its role in matured neurons (Miao et al., 1997; Traiffort et al., 1998, 1999; Ho and Scott, 2002). This is the first evidence that nerve axotomy induces upregulation of both Shh and Smo. This coordinated expression pattern, of both the ligand and receptor, in adult motor neurons strongly suggests that Shh may act on motor neurons in an autocrine manner. Another possibility is that Shh may act on their surrounding cells such as astrocytes, microglial cells, and Schwann cells to release molecules mediating motor neuron survival. Although the precise molecular circuit still remains unclear, our findings imply the novel function of Shh in matured motor neurons altering its expression level after nerve injury. Recently, new roles of Shh have been intensively characterized. In the adult hippocampus, Shh has been shown as a regulator of stem cell proliferation (Lai et al., 2003). It was also reported that Shh can directly attract spinal cord axons and commissural axons as a chemoattractant (Trousse et al., 2001; Charron et al., 2003). It is interesting that, in addition to its earlier role in development, Shh may function later in a novel role in the adult brain. Additional studies will confirm how widely this key molecule is used not just in the embryo but also in the adult nervous system.

It has not been fully elucidated why axotomy of neonatal rats results in the cell death of motor neurons, whereas axotomy of adult rats results in successful regeneration (Graeber et al., 1998). Although a number of molecules involved in the repair process have been identified and characterized, the precise timing of these cascades may be critical to understand the process of nerve regeneration. We found a quite contrasting result that only adult rat axotomy induces the upregulation of Shh and Smo expression, whereas neonatal rat axotomy does not. Thus, we tested the gain of function of Shh on axotomized neonatal rats. We observed that AdV-mediated gene transfer of Shh after axotomy of neonatal rats transiently endows the axotomized motor neurons with the ability to survive. The modified AdV used in our studies infects cells through a receptor-mediated mechanism. AdV vectors can infect cells *in vivo* to express a high level of the transgene within hours after infection (Moriyoshi et al., 1996; Verma and Somia, 1997). In somatic tissues, the expression sometimes lasts for 5–10 d after infection, attributable in part to viral-induced immune responses of the recipients. In fact, there are also reports describing a relatively limited period of gene transfer via AdV vectors (Baumgartner and Shine, 1998; Hottinger et al., 2000). In our experiments, it is possible that the survival-promoting effect of Shh is influenced by adenovirus toxicity, limiting the number and the duration of survived neurons. The modification of viral vector and transfer methods is necessary to improve the efficacy of regeneration. Alternatively, the development of pharmacological agonist–antagonist Shh signaling will be also beneficial if oral treatment is effective, as described recently (Chen et al., 2002a; Machold et al., 2003).

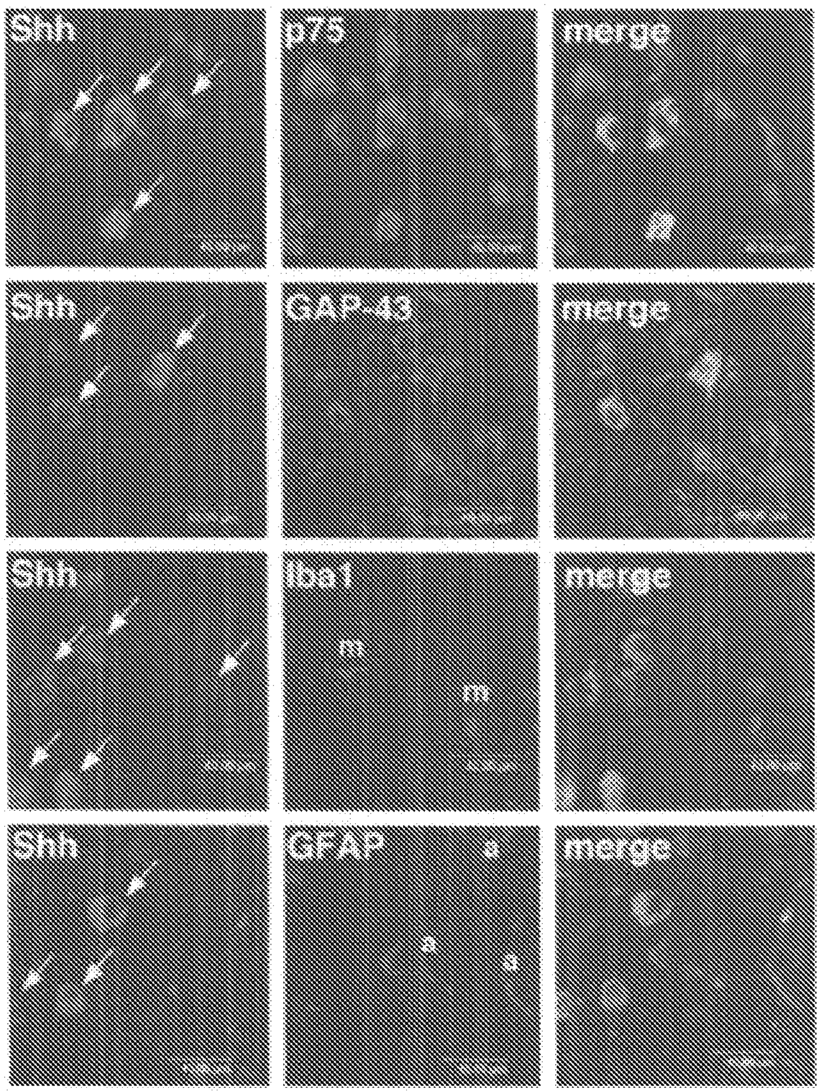

Figure 5. Cellular localization of adenovirus-mediated Shh expression in the lesioned facial nuclei of neonatal rats. The immunoreactivities of the anti-Shh monoclonal antibody were visualized with Alexa Fluor 488 (green, left). The neuronal (p75 and GAP-43) and non-neuronal (m, Iba1 for microglia and macrophages; a, GFAP for astrocytes) markers were visualized with Alexa Fluor 594 (red, middle). Shh immunoreactivities (arrows) were observed in the cell bodies of p75-positive or GAP-43-positive neurons but not detected in the cell bodies of Iba1-positive (m) or GFAP-positive (a) cells. Scale bar, 40 μm.

We assessed the cellular expression of AdV–Shh in the lesioned facial nucleus. Immunoreactivities of Shh were strongly observed in the cell bodies of facial motor neurons but were not detected in the cell bodies of astrocytes or microglia. Based on our current observations, the selective upregulation of Shh and Smo in axotomized motor neurons may serve in multiple pathways. The Shh may function as a survival factor per se in an autocrine manner. The motor neurons in response to the Shh may influence surrounding cells through unidentified molecules. Alternatively, the secreted Shh may directly affect surrounding cells, although, at least in our experimental conditions, the expression of Ptc1 and Smo was under the detection level in glial cells. In this paper, we described the immunoreactivities of Iba1 that resides in microglia, and microglia-derived macrophages were detected in the cells adjacent to Shh-positive motor neurons. Phagocytic microglia surround the injured neurons to remove the lethally damaged neurons to preserve functional and structural architects of the lesioned nervous system (Graeber et al., 1998). Additional studies on Shh signaling will clarify how precisely the cellular network is preserved in nerve regeneration.

Although exogenous Shh promotes the transient survival of axotomized motor neurons of neonatal rats, this does not answer the question of whether the endogenous Shh is involved in the regeneration of axotomized neurons. We used the steroidal alkaloid cyclopamine, which is known to inhibit the Shh signaling through the specific binding of cyclopamine and Smo, altering the signal transduction of Smo (Chen et al., 2002b). In the axotomized adult rats, the application of cyclopamine adjacent to the nerve cut site significantly reduced the number of Nissl-positive motor neurons in a dose-dependent manner, whereas its inactive analog tomatidine had no effect. Taken together with our present

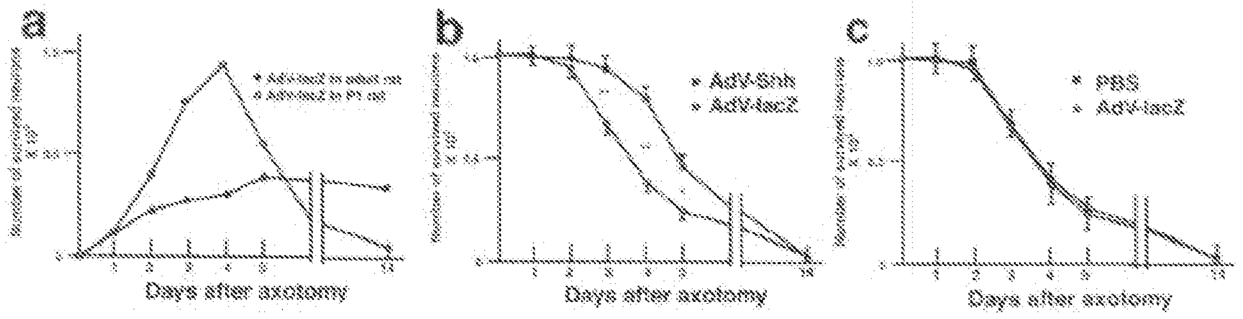

Figure 6. a, Efficacy of adenovirus-mediated overexpression of lacZ after axotomy in P1 and adult rats in the lesioned motor neurons. The pups were treated for 4 d after axotomy after the implantation of gelfoams containing AdV–lacZ. The number of lacZ-positive neurons was counted. In the neonatal rats, more lacZ-positive neurons were observed than in the adult rats by the adenovirus gene transfer. The number of Nissl-positive survived neurons was counted from the sections covering bilateral facial nuclei. Results of three independent experiments were subjected to statistical analysis (Student's t test; $^*p < 0.05$; $^{**}p < 0.001$). Shh–adenovirus significantly potentiates the survival of axotomized motor neurons from 1 to 5 d after axotomy and adenovirus application (b). An application of adenovirus after axotomy did not influence the number of survived neurons compared with PBS application (c).

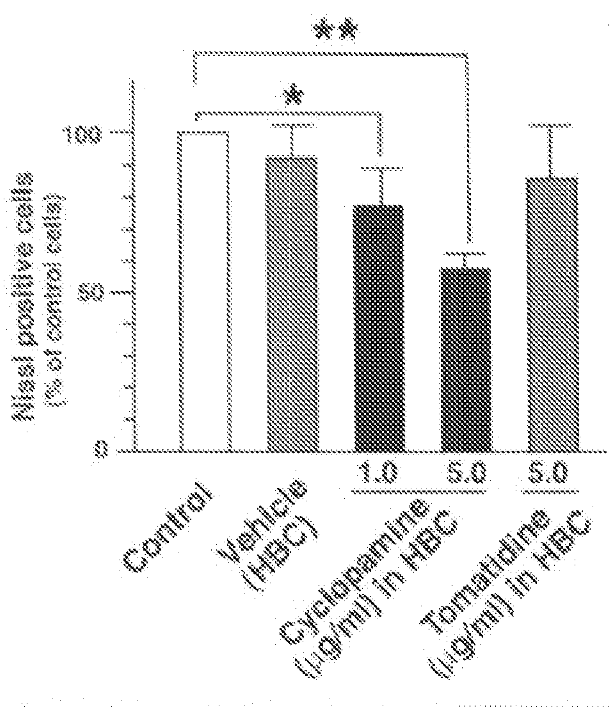

Figure 7. Inhibition of Shh signaling in axotomized adult rats. The cyclopamine application resulted in the loss of motor neurons in vivo. Bilateral facial nerves were axotomized and a Gelfoam soaked with cyclopamine (1.0 or 5.0 μg/ml), with tomatidine (5.0 μg/ml), or with vehicle (45% w/v HBC) was implanted in each side. The total number of Nissl-positive cells was counted and compared between the implanted side and the control side. Five independent animals were operated on and statistically analyzed by Student's t test ($^*p < 0.05$; $^{**}p < 0.01$).

result. It is strongly suggested that endogenous Shh plays an important role in the adult brain, and this represents one of the few demonstrations of Shh function in the adult brain.

Our findings suggest that in addition to its earlier role in directing cellular fates in development, Shh functions as a regulatory role after nerve injury. This adds to a rapidly growing list of factors that have been identified as key molecules in nerve regeneration. Although considerable progress has been made in recent years in defining the genetic and molecular control of nerve regeneration, characterization in detail of molecular cascades has lagged. Our results in vivo have a therapeutic implication of Shh for regeneration of neuronal tissues after injuries. Based on our in vivo studies, besides its well characterized effects during development, we propose the de novo function of Shh in the regeneration of injured motor neurons.

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Suppression of the Shh pathway using a small molecule inhibitor eliminates medulloblastoma in *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice

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Summary

Medulloblastoma is the most common malignant pediatric brain tumor. Current treatment is associated with major long-term side effects; therefore, new nontoxic therapies, targeting specific molecular defects in this cancer, need to be developed. We use a mouse model of medulloblastoma to show that inhibition of the Sonic Hedgehog (Shh) pathway provides a novel therapy for medulloblastoma. A small molecule inhibitor of the Shh pathway, HhAntag, blocked the function of Smoothened in mice with medulloblastoma. This resulted in suppression of several genes highly expressed in medulloblastoma, inhibition of cell proliferation, increase in cell death and, at the highest dose, complete eradication of tumors. Long-term treatment with HhAntag prolonged medulloblastoma-free survival. These findings support the development of Shh antagonists for the treatment of medulloblastoma.

Introduction

Research in molecular oncology has benefited from the advent of mouse models of human cancer (Hesselager and Holland, 2003; Van Dyke and Jacks, 2002). Genetically engineered mouse strains have been used to investigate tumor etiology and to elaborate the molecular pathways that contribute to human cancer. However, the greatest potential application of such models is to pioneer the development of novel therapies that are specifically targeted against growth control pathways altered in cancer. For this purpose, it is necessary that mouse models recapitulate both the genetics and the biology of the corresponding human disease. It is also critical that the tumor incidence is high and the age of onset is predictable, to ensure that cohorts of mice bearing tumors can be identified and treated with potential anticancer drugs. Previously, we developed a high incidence mouse model for the pediatric brain tumor medulloblastoma (Wetmore et al., 2001). Medulloblastoma, a primitive neuroectodermal tumor, arises in the cerebellum and accounts for approximately 20% of all pediatric brain tumors. Current treatment involves surgical resection, chemotherapy, and radiation of the craniospinal axis (Ellison et al., 2003). Although the overall 5 year survival rate is approximately 70%, treatment is associated with long-term side effects including ataxia, neuroendocrine problems, intellectual deterioration, and neuropsychological difficulties (Chintagumpala et al., 2001; Ellison et al., 2003). There is a critical need for new, nontoxic therapies for medulloblastoma.

The first indication that alteration in the Shh pathway contributes to medulloblastoma formation was the discovery that germline mutations in the Shh receptor, *Patched-1* (*PTCH1*), underlie the human developmental disorder Gorlin syndrome (Gorlin, 1995; Hahn et al., 1996; Johnson et al., 1996). Gorlin syndrome, also known as Basal Cell Nevus Syndrome, is characterized by large body size, developmental and skeletal abnormalities, fibromas of soft tissue, radiation sensitivity, basal cell carcinoma, and increased incidence of medulloblastoma (Goodrich and Scott, 1998; Kimonis et al., 1997). Heterozygous loss of *PTCH1* occurs in approximately 10% of sporadic human medulloblastoma (Ellison et al., 2003; Wechsler-Reya and Scott, 2001), and mutations in downstream components of the Shh pathway, Smoothened (*SMOH*) and Suppressor of Fused (*SUFU*), which lead to activation of the pathway, have also been described (Reifenberger et al., 1998; Taylor et al., 2002). In mice, heterozygous mutation of *Patched-1* (*Ptc1*) is associated with an approximately 20% incidence of medulloblastoma over a period of 1 year (Corcoran and Scott, 2001; Goodrich et al., 1997; Wetmore et al., 2000). We accelerated the incidence and decreased the latency of medulloblastoma by crossing *Ptc1*$^{+/-}$ mice with *p53*$^{-/-}$ mice (Wetmore et al., 2001). *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice develop medulloblastoma and most die from brain tumors within 12 weeks of birth. *P53* is mutated in approximately 10% of human

SIGNIFICANCE

Therapy for pediatric cancers of the CNS has not improved significantly in the last three decades. This is partly due to the absence of adequate model systems for testing novel therapies. Current models are based on xenografts, allografts, or in vitro culture of human tumor cells. In these models, the cancer cells are removed from their normal environment, undergo selective pressure, and may not be representative of the original tumor. Cancer models should recapitulate the causative genetic lesions and the anatomical location and developmental time frame of the corresponding human disease. Here, we use such a model to test a novel therapy for medulloblastoma; we show that suppression of the Sonic Hedgehog pathway can be an effective treatment for this tumor.

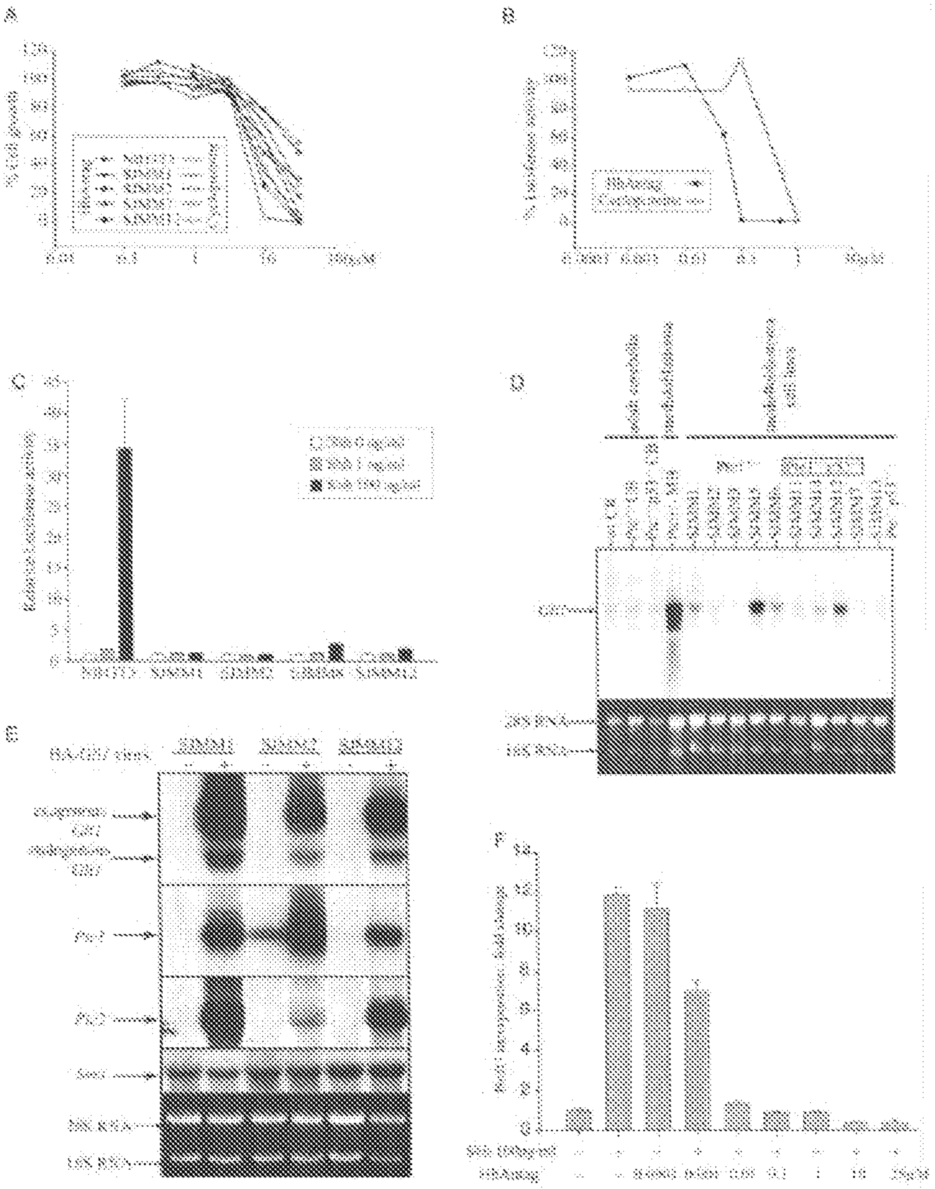

Figure 1. The Shh pathway is downregulated in cultured medulloblastoma cells

A: HhAntag and cyclopamine inhibit growth of cultured NIH3T3 and medulloblastoma cells at similar concentrations. The graph shows the percentile change in cell growth, as determined by Alamar blue staining, following treatment of cells with increasing concentrations of HhAntag or cyclopamine. The medulloblastoma cell cultures, SJMM1 and SJMM2, were derived from Ptc1$^{+/-}$ tumors, whereas SJMM8 and SJMM12 were derived from Ptc1$^{+/-}$ p53$^{-/-}$ tumors.

B: The Shh pathway was activated in NIH3T3 cells containing a Gli-luciferase reporter by treatment with 100 ng/ml ShhN, and the inhibitory effects of HhAntag and cyclopamine were compared. HhAntag is ~10 times better than cyclopamine at inhibiting Shh activity. Note that the concentration of HhAntag required to inhibit cell growth is approximately 1000-fold higher than that needed to suppress Shh activity.

C: Shh induces Gli-luciferase activity in NIH3T3 cells but not in medulloblastoma cell cultures. Medulloblastoma cultures and NIH3T3 cells transfected with a Gli-luciferase reporter plasmid were treated with increasing doses of Shh.

D: Gli1 is highly expressed in medulloblastoma in vivo but is downregulated in cell lines derived from tumors. Total RNA, isolated from normal cerebella, medulloblastoma, or medulloblastoma cultures, was analyzed by hybridization to a Gli1 probe. Ethidium bromide staining of the 28S and 18S rRNA is shown as a loading control.

E: Gli1 can activate target genes in medulloblastoma cells. Cells were infected with a retroviral vector expressing an HA-tagged full-length Gli1 cDNA. RNA was isolated and analyzed by hybridization to probes specific for Gli1, Ptc1, Ptc2, and Smo. Ethidium bromide staining of the 28S and 18S rRNA is shown as a loading control.

medulloblastoma, and the P53 pathway is defective in a larger subset of tumors (Ellison, 2002; Frank et al., 2004; Kleihues et al., 1997). In addition, germline mutations of *P53* are associated with medulloblastoma in Li-Fraumeni syndrome (Malkin et al., 1990). Thus, *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice provide a useful model to develop novel therapies for brain tumors, targeted against the Shh pathway.

During brain development, Shh released by migrating Purkinje cells binds to Ptc1 present on granule neuron precursor (GNP) cells in the external germinal layer (EGL) of the cerebellum (Wechsler-Reya and Scott, 2001). This interaction causes derepression of Smoothened (Smo) (Taipale et al., 2002) and increased expression of transcription factor *Gli1* and proliferation of GNP cells. The Shh pathway is critical during embryogenesis but is downregulated after early postnatal development in most tissues, including the brain. In contrast, more than 30% of human medulloblastoma exhibit high levels of *GLI1* expression (Lee et al., 2003), indicating that abnormal activation of the Shh pathway is important in a subset of pediatric brain tumors. Thus, the Shh pathway is a potential therapeutic target for development of novel treatments for medulloblastoma.

Cyclopamine, an alkaloid isolated from the corn lily *Veratrum californicum* (Binns et al., 1963; Keeler and Binns, 1968), has been shown to suppress the response of target tissues to Shh (Cooper et al., 1998) by binding to Smo (Chen et al., 2002). Recent observations suggest that cyclopamine inhibits the growth of cultured medulloblastoma cells and allografts by inhibiting Smo (Berman et al., 2002). However, cyclopamine is not a good drug candidate for treatment of medulloblastoma because it has a relatively low affinity for Smo. High-throughput, cell-based screening assays identified several small molecule inhibitors of the Shh pathway (Williams et al., 2003). One of these compounds, a benzimidazole derivative, Hh-Antag691, here referred to as HhAntag (Gabay et al., 2003), exhibited higher affinity for Smo than cyclopamine, blocking Shh function at >10-fold lower concentrations. HhAntag penetrates the blood-brain barrier after oral delivery, making it an ideal candidate for treatment of brain tumors caused by increased activity of Smo. Therefore, we selected HhAntag to treat medulloblastoma in *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice. We found that HhAntag inhibited *Gli1* expression in a dose-dependent manner in tumor tissue in vivo. Treatment of tumor-bearing mice twice daily with HhAntag at 20 or 100 mg/kg of body weight, by oral gavage, for 4 days, inhibited tumor cell proliferation and increased apoptosis. Twice daily treatment of mice for 2 weeks with 20 mg/kg of HhAntag resulted in reduction of tumor volume, and complete elimination of tumors was observed in mice treated with 100 mg/kg HhAntag. In longer-term studies, 100 mg/kg HhAntag given once a day significantly enhanced brain tumor-free survival. These results suggest that HhAntag may be beneficial for treatment of medulloblastoma and other tumors in which the Shh pathway is deregulated.

Results

HhAntag blocks Shh signaling at concentrations that do not affect cell growth

As a first step in analyzing the ability of HhAntag to block medulloblastoma growth, we treated cultured tumor cells derived from *Ptc1*$^{+/-}$ and *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice as well as NIH3T3 cells with increasing concentrations of HhAntag or cyclopamine. We measured the effects on cell growth, as determined by Alamar blue staining, and found that both compounds exhibited similar IC_{50} values in growth assays (10–30 μM), with growth of some medulloblastoma cultures inhibited more readily by cyclopamine than HhAntag (Figure 1A). Similar results were obtained when we used cell counts as a measure of proliferation. Next, we looked at the concentration of HhAntag and cyclopamine required to suppress the Shh pathway. We transfected NIH3T3 cells with a *Gli*-luciferase reporter construct and measured inhibition of the Shh pathway by the two compounds following maximal stimulation by Shh (Figure 1B). HhAntag was significantly more potent than cyclopamine at suppressing Shh signaling; it exhibited an EC_{50} of 0.04 μM compared to 0.5 μM for cyclopamine (Figure 1B). Surprisingly, the drug concentrations required to block cell growth were higher than those required to inhibit Smo activity by approximately 100- and 1000-fold for cyclopamine and HhAntag, respectively. Indeed, both HhAntag and cyclopamine were able to completely suppress Shh activation of *Gli*-luciferase in NIH3T3 cells at concentrations that did not affect cell growth. These results support the selection of HhAntag as a potent Smo inhibitor for preclinical studies on medulloblastoma. However, they also raise the concern that growth inhibition in cell culture may not be related to inhibition of Smo, as the concentrations that suppressed the Shh pathway were 100- to 1000-fold lower than those that inhibited cell growth.

The Shh pathway is not active in cultured medulloblastoma cells

To investigate the status of the Shh pathway in cultured medulloblastoma cells, we treated NIH3T3 cells and medulloblastoma cultures containing a *Gli*-luciferase construct with Shh (Figure 1C). Although NIH3T3 cells exhibited a strong dose-dependent increase in luciferase activity in response to Shh, all of the medulloblastoma cultures failed to respond. Previously, we reported that medulloblastomas express high levels of *Gli1*, indicating that the Shh pathway is active in tumors in vivo (Wetmore et al., 2001). However, examination of mRNA levels in cultured medulloblastoma cells revealed that *Gli1* mRNA was much reduced or absent in vitro (Figure 1D). To ensure that the downstream components of the Shh pathway were intact in medulloblastoma cell cultures, we confirmed the presence of normal *Smo* and *Ptc1* mRNA by sequencing cDNA derived from tumor cell RNA. In addition, we demonstrated that ectopic expression of *Gli1*, using a retroviral vector, increased expression of the endogenous target genes *Gli1*, *Ptc1*, and *Ptc2* (Figure 1E). These findings demonstrate that the Shh pathway is suppressed in

F. HhAntag efficiently blocks Shh-induced proliferation of GNP cells in a dose-dependent manner. GNP cells, isolated from postnatal day 6 mice, were treated with 100 ng/ml Shh in the presence of increasing concentrations of HhAntag. Cell proliferation was measured by determining the fold difference in BrdU incorporation, relative to incorporation in the absence of Shh. Note, much lower concentrations of HhAntag block growth of GNP cells than those required to block growth of medulloblastoma cells in culture.

cultured medulloblastoma cells at a step between Smo and Gli1 and they imply that growth inhibition caused by exposure to cyclopamine and HhAntag in vitro (Figure 1A) is not mediated by inactivation of Smo. In contrast to the high concentration of drug required to inhibit growth of medulloblastoma and NIH3T3 cells, HhAntag efficiently blocked Shh-mediated proliferation of GNP cells at the same low concentrations that inhibited Gli-luciferase activity (Figure 1F). Thus, it is possible that additional factors present in the intracranial environment and absent in culture are needed to maintain the ability of medulloblastoma cells to respond to Shh.

HhAntag inhibits the Shh pathway in medulloblastoma cells in vivo

The results presented above indicate that Smo activity is not required for proliferation of medulloblastoma cells in culture. To determine if Smo activity can be blocked in tumor cells in vivo, we analyzed *Gli1* expression in *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice treated with HhAntag. Cohorts of mutant mice were treated with HhAntag, administered twice daily by oral gavage, for 4 days, at 20 or 100 mg/kg of body weight. To control for nonspecific effects, we also treated a cohort of mutant mice with vehicle. Treatment was initiated in 6-week-old *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice, as they contain substantial tumor mass at this age. At the end of the dosing regimen, mice were euthanized and total RNA was isolated from tumor tissue together with the entire cerebellum. This approach reduces sampling errors that would be introduced by attempting to dissect tumor tissue free from normal cerebellum. Tumors in *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice arise in very young mice, and they are intimately associated with normal cerebellar structure. By combining tumor tissue and the complete cerebellum, we ensure that the normal component of each sample is constant and the differences observed in gene expression reflect changes in tumor cells. We also measured the level of HhAntag in the remaining brain tissue to confirm that the compound penetrated the blood-brain barrier. The mice treated with 20 and 100 mg/kg of HhAntag contained an average of 0.38 and 2.8 mg/kg of drug, respectively, and no compound was detected in vehicle-treated mice (Figure 2A).

Quantitative real-time PCR analysis of *Gli1* expression levels revealed extensive variation among control samples (Figure 2A). This was expected, as the time of tumor development and tumor growth rate vary among individual mice in this spontaneous brain tumor model. Therefore, we used the Exact Kruskal-Wallis test to determine whether the results reflected significant differences in gene expression among the different treatment groups (Figure 2B). HhAntag effectively suppressed *Gli1* mRNA levels in a dose-dependent manner in vivo (Figures 2A and 2B). In addition to *Gli1*, we examined the levels of several other genes (*Sfrp1*, *Math1*, and *Ptc2*) previously identified to be highly expressed in *Ptc1*$^{+/-}$*p53*$^{-/-}$ medulloblastoma (Lee et al., 2003). We found a dose-dependent decrease in mRNA levels of these genes in response to HhAntag (Figure 2B). As a control, we examined expression of *Reln*, which is present in GNP and tumor cells (Wetmore et al., 2000) but is not known to be under control of the Shh pathway. The *Reln* levels were not affected by drug treatment (Figure 2B). Surprisingly, transcription of the wild-type allele of *Ptc1*, which like *Gli1* is a target of Shh signaling, was unaffected by drug treatment (Figure 2B). Previously, we demonstrated that both the normal and the mutated *Ptc1* alleles are expressed in *Ptc1*$^{+/-}$ medulloblastoma cells at levels comparable to that in normal developing cerebellum (Wetmore et al., 2000). This agrees with the original report from Goodrich et al. (1997) demonstrating expression of the mutated *Ptc1* allele in tumor cell by β-gal staining. Thus, although *Ptc1* expression has been used to monitor the response of mouse medulloblastoma cells to Smo inhibition in vitro (Berman et al., 2002), it appears that *Ptc1* is not a target of the Shh pathway in these tumor cells in vivo.

The reduction in mRNA levels of Shh pathway target genes observed in the short-term study was not simply due to loss of tumor mass following treatment with HhAntag, as histological analyses of cerebella from treated mice indicated that tumors were present in all treated mice. To confirm that gene expression was decreased in tumor cells in vivo, we conducted in situ hybridization analyses of brain sections using probes specific for *Gli1*, *Sfrp1*, *Math1*, *Ptc1*, *Ptc2*, and *Reln* genes (Figure 3). Consistent with the quantitative PCR analysis, we found that *Gli1*, *Sfrp1*, *Math1*, and *Ptc2* were expressed mostly in the tumor cells and not in the adjacent normal cerebellum. There was a marked decrease in expression of all of these genes in response to 20 mg/kg of HhAntag and a complete absence of expression following treatment with 100 mg/kg, despite the presence of substantial tumor mass (Figure 3). When the decrease in gene expression levels between vehicle and 100 mg/kg HhAntag-treated mice (7- to 300-fold changes, depending on the gene) is compared with the decrease observed in tumor cell density (1.8-fold), it is clear that the loss of gene expression is not simply due to the reduction in cell number following treatment. In agreement with the real-time PCR data, wild-type *Ptc1* mRNA was present at relatively low levels in the tumor tissue, and its expression did not change after drug treatment (Figure 3). The control gene, *Reln*, was still expressed at high levels in tumor present in the drug-treated mice. Thus, HhAntag selectively suppressed Shh pathway target genes in vivo by blocking Smo signaling in medulloblastoma cells, with complete suppression at 100 mg/kg given twice a day.

HhAntag affects morphology and growth of *Ptc1*$^{+/-}$*p53*$^{-/-}$ medulloblastoma

Since HhAntag effectively blocked Smo activity in mice treated for 4 days, we analyzed the morphology of treated tumors. Neuropathological examination readily distinguished among tumors from untreated and 20 or 100 mg/kg of HhAntag-treated mice (Figure 4A). Although all tumors were composed of small round cells with oval to carrot-shaped hyperchromatic nuclei and minimal surrounding cytoplasm, those in the treated mice less often formed large, confluent tumor masses. The lesions in treated mice more often manifest as small collections of individual tumor cells in the superficial confines of the cerebellar molecular layer. The most striking difference observed among the three groups was a dramatic decrease in mitotic activity (Figure 4B). Immunohistochemical staining with the Ki67 marker for cell proliferation confirmed the observation that HhAntag decreased the proliferative index in tumor tissues ($p < 0.0001$, Exact Kruskal-Wallis) (Figure 4C). We also observed more cell death in tumors from treated mice, as indicated by increased numbers of pyknotic nuclei (Figure 4B) and by TUNEL assay ($p = 0.0402$, Exact Kruskal-Wallis) (Figure 4C). The influx of macrophages and microglia into the drug-treated tumors (as determined by Mac2 immunohistochemistry) increased from

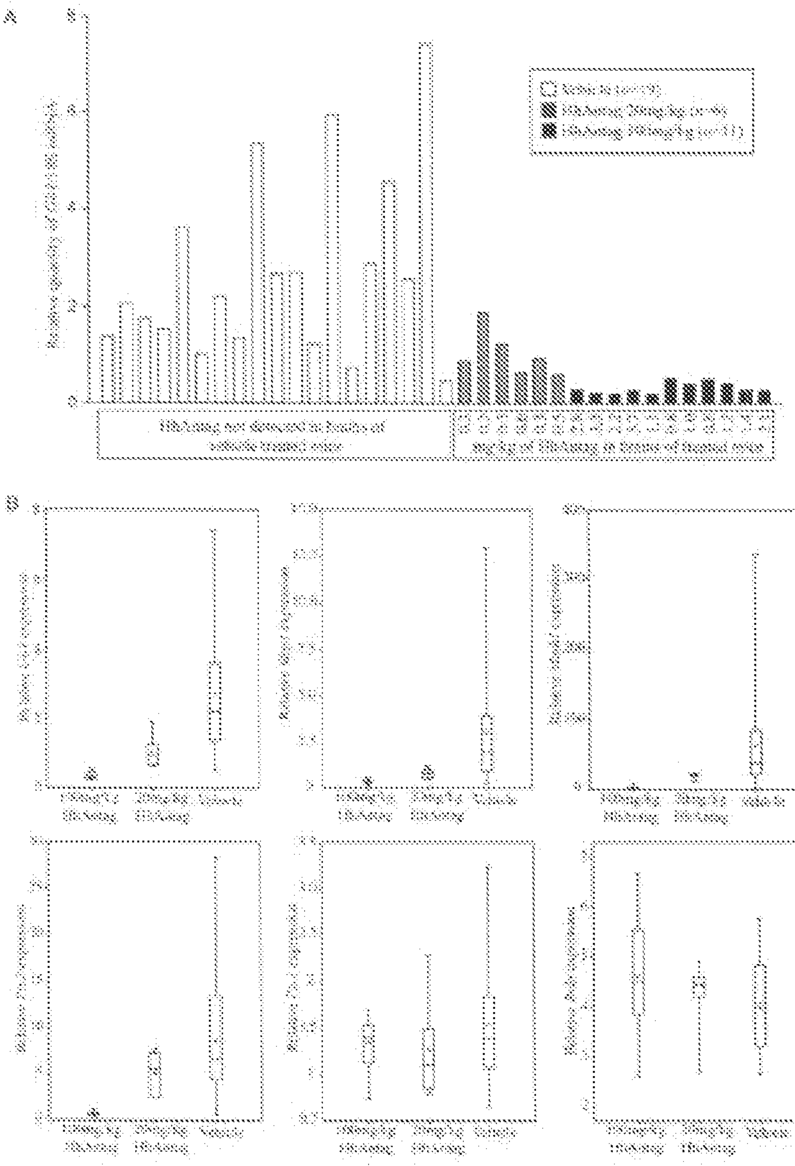

Figure 3. HhAntag suppresses the Shh pathway in mouse medulloblastoma in vivo

A: Real-time PCR analyses of total RNA isolated from tumor-bearing cerebella showing Gli1 expression in mice treated twice daily for 4 days with HhAntag at 20 or 100 mg/kg or with vehicle alone. The amount of HhAntag present in each treated brain, 4 hr after the last dose, is indicated under each of the bars.

B: Box plots derived using Exact Kruskal-Wallis test to analyze gene expression in the treated brains showing that HhAntag suppressed Gli1, Sfrp1, Math1, and Ptc2, but not Ptc1. [illegible], a gene expressed in medulloblastoma, but not under control of the Shh pathway, was analyzed as a control. Levels of mRNA for all assayed genes were within the linear range of a standard curve derived by amplifying each gene from serially diluted RNA, isolated from an independent source.

2.5% of Mac2-positive cells in vehicle-treated tumors to 5% and 7% in tumors treated with 20 and 100 mg/kg of HhAntag, respectively. Macrophage infiltration is associated with damage or loss of cells and it is not necessarily indicative of necrosis. In fact, necrosis was not observed in any of the tumors treated with HhAntag. HhAntag treatment also caused a dose-dependent increase in expression of the astrocyte marker, GFAP, which is characteristic of reactive gliosis often associated with CNS trauma. Taken together, the data presented above indicate that even a short-term treatment with HhAntag inhibited tumor growth.

After completion of treatment, we observed that, in the vehicle group, four out of five mice exhibited an overwhelming tumor burden and only one mouse had a small tumor. In contrast, in the cohort treated with 20 mg/kg of HhAntag, one mouse had a very large tumor, three had small tumors, and one had almost no tumor, whereas in the 100 mg/kg treatment group, one mouse had a large tumor, one had a medium-sized tumor, and three mice showed almost no tumor burden. Representative sagittal sections, taken through the largest tumor cross-section, are shown in Supplemental Figure S1. Although the short-term treatment with HhAntag appeared to inhibit tumor growth, the differences in tumor size between vehicle- and drug-treated mice were not dramatic. Therefore, we designed a study to

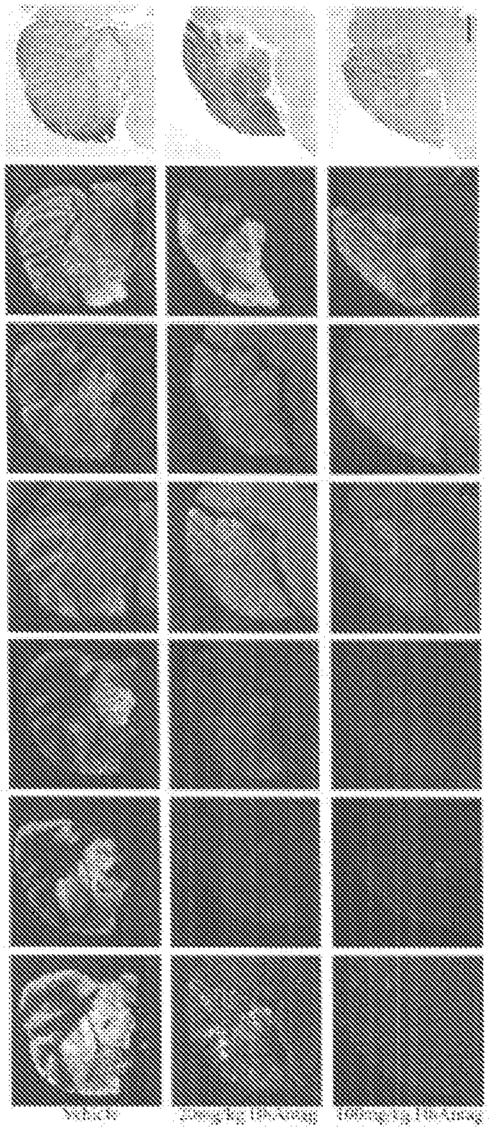

Figure 3. Treatment of *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice with HhAntag suppresses *Gli1*, *Sfrp1*, *Math1*, and *Ptc2*, but not *Ptc1* in medulloblastoma

In situ hybridization analyses were performed using radiolabeled probes on brain tissue from *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice. Expression of *Gli1*, *Sfrp1*, *Math1*, and *Ptc2* genes was downregulated after 4 days of treatment, twice daily with 20 mg/kg of HhAntag, and completely suppressed following treatment with 100 mg/kg HhAntag. *Ptc1* mRNA was present at low levels in the tumor and in the normal Purkinje cell and internal granule cell layers. Treatment with HhAntag did not alter the pattern or intensity of the *Ptc1* signal. *Zic1* mRNA, highly expressed in medulloblastoma, did not change following treatment. The panels on the far right show adjacent paraffin sections of the tumor-bearing cerebella, stained with hematoxylin and eosin, tumor regions outlined. Scale bar equals 800 μm.

determine the effect of HhAntag on tumor volume in *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice treated for the extended period of 2 weeks.

HhAntag eliminates tumor mass in *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice

To determine the effects of HhAntag on tumor growth, mice were treated for 14 days, twice daily, at either 20 or 100 mg/kg, or with vehicle alone. Treatment was initiated when mice were 3 weeks old, at which age all *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice exhibit discernible tumor mass but not of a size that could adversely affect their health and short-term survival. At the end of the treatment period, brains were prepared for histological examination. We found that all the vehicle-treated mice had substantial tumor mass, whereas mice treated with 20 mg/kg of HhAntag had much smaller tumors and no tumor mass could be detected in the 100 mg/kg treatment group (Figure 5A). We used three-dimensional reconstruction analysis to measure tumor volumes by tracing sections at 150 μm intervals through the cerebellum (Figures 5B and 5C). Both the remaining normal IGL and the tumor areas were traced, and the tracings were aligned using the IGL as a guide to reconstruct tumor volume in 3D (Figures 5D and 5E). This is illustrated as a 3D computer-generated image with the IGL in blue (Figure 5D) and the tumor volume in several colors (Figures 5D and 5E). The IGL volume was subtracted in Figure 5E to clearly identify the total tumor volume in this example. Quantitative analysis revealed a marked decrease of tumor size in mice treated with 20 mg/kg HhAntag and a complete elimination of measurable tumor volume following treatment with 100 mg/kg HhAntag (Figure 5F). There was variation in the tumor mass present in the untreated cohort (5.8–34.8 mm^3) and in the 20 mg/kg treated mice (0.11–7.56 mm^3). The range of volumes was greater in untreated mice, as indicated by the standard deviation of 12.8 compared to 2.8 for the 20 mg/kg treated mice. The overall reduction in tumor volumes following treatment with HhAntag was examined using the Exact Kruskal-Wallis test; the Monte Carlo estimate of the p value (<0.0001) suggests a highly statistically significant differences in tumor volume distribution among the three treatment groups. We also found that there was a dose-dependent effect of HhAntag on tumor volume as indicated by the Exact Wilcoxon-Mann-Whitney test ($p = 0.0159$). There was an approximate 7-fold reduction in tumor volume between the untreated mice and mice treated with 20 mg/kg HhAntag (from an average of 19 mm^3 to 2.5 mm^3). Strikingly, complete tumor regression was observed following treatment of mice with 100 mg/kg HhAntag. In two of the four mice treated, no tumor remained at all. However, we did observe small lesions, indicating evidence of regression in the most lateral aspect of the cerebellum of the other two mice (Figure 5G). These areas showed no evidence of proliferation and they contained scattered apoptotic bodies and focal reactive changes, particularly lymphocyte infiltrate from leptomeningeal blood vessels. The lesions were confined to the molecular layer and the subpial zone. Thus, HhAntag, given twice daily at 100 mg/kg, effectively eliminated medulloblastoma in *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice.

HhAntag prolongs brain tumor-free survival of *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice

To determine if HhAntag could prolong survival of *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice, we treated 20 5-week-old mice with either 100 mg/kg of the drug or vehicle, once daily for an extended period. The dose was lower than used in the previous studies because of limited

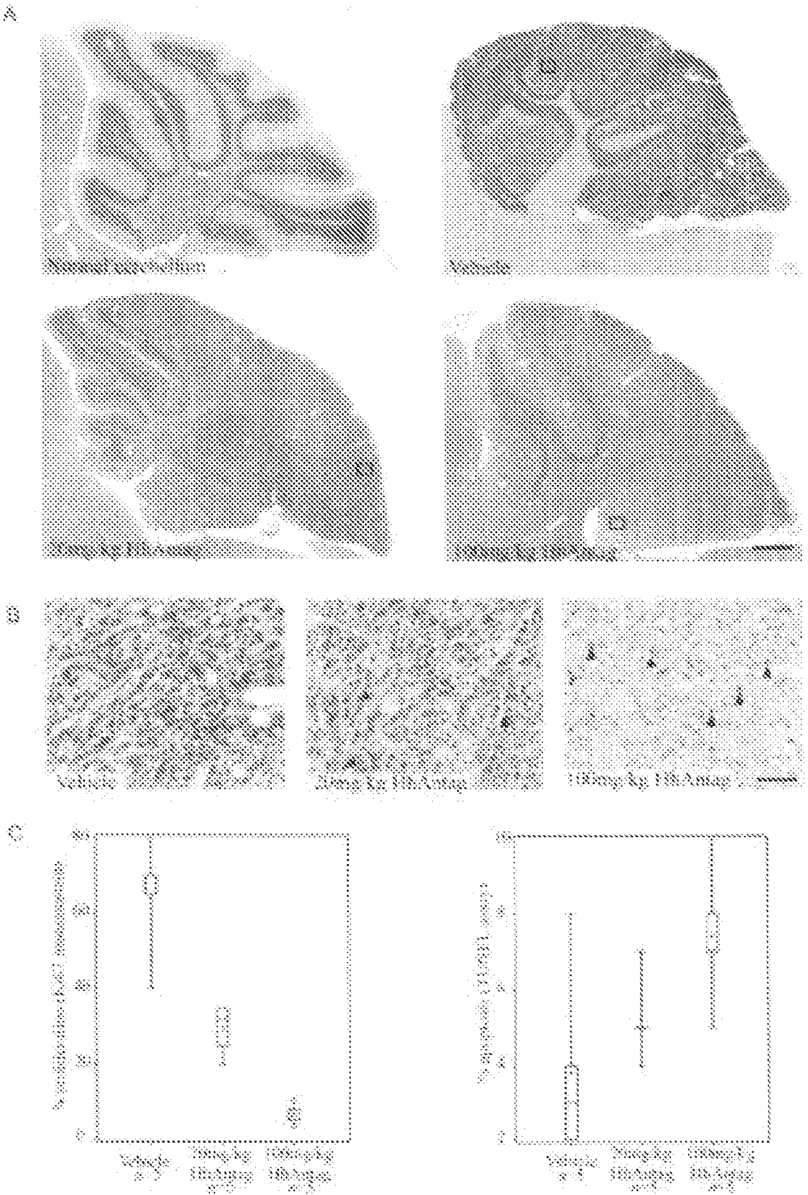

Figure 4. HhAntag inhibits proliferation, increases apoptosis, and alters morphology of medulloblastoma cells in vivo

A: Sections of brain tissue from mice treated twice daily with vehicle or HhAntag at 20 or 100 mg/kg, at low magnification. Scale bar equals 500 μm.

B: High magnification of **A**. Scale bar equals 50 μm. The locations of the regions examined at high magnification are indicated by black boxes. Note the increase in apoptotic bodies (black arrowheads) and the decrease in mitotic figures (white arrowheads) associated with HhAntag treatment. All sections were stained with hematoxylin and eosin.

C: Quantitative analyses of proliferation and apoptosis in tumors are presented as box plots. The data were obtained by counting Ki67 (proliferation) and TUNEL (apoptosis) positive cells in the drug- and vehicle-treated mice. The data were analyzed using the Exact Kruskal-Wallis test, with Monte Carlo estimates of p values of <0.0001 for proliferation and 0.0402 for apoptosis, suggesting statistically significant difference among the three treatment groups.

availability of the HhAntag compound and the concern that prolonged, twice daily delivery of the drug by oral gavage could result in injury. Also due to the limited availability of HhAntag, four of the ten mice receiving HhAntag were removed from drug after 50 days of treatment. All mice treated with vehicle alone developed clinical signs of brain tumors, such as domed head, ataxia, and tilting of the body, and were euthanized between 4 and 74 days after the start of treatment. In contrast, drug-treated mice survived significantly longer (38 to 147 days), and only five mice were euthanized due to brain tumors (Figure 5). Three of these had received HhAntag for only 50 days. The remaining five mice in the treated cohort were euthanized after exhibiting symptoms of other tumors typical of *p53* null mice, including thymic lymphomas, soft tissue sarcomas, and teratomas. However, each of these mice also showed evidence of small medulloblastomas when the brain was removed and examined. These results indicate that treatment of mice with 100 mg/kg of HhAntag once daily did not completely eliminate medulloblastoma. This is most probably because the dose of drug used, while sufficient to retard tumor growth, was not high enough to cause total regression. Nevertheless, analyses of the data revealed that HhAntag significantly extended the period of brain tumor-

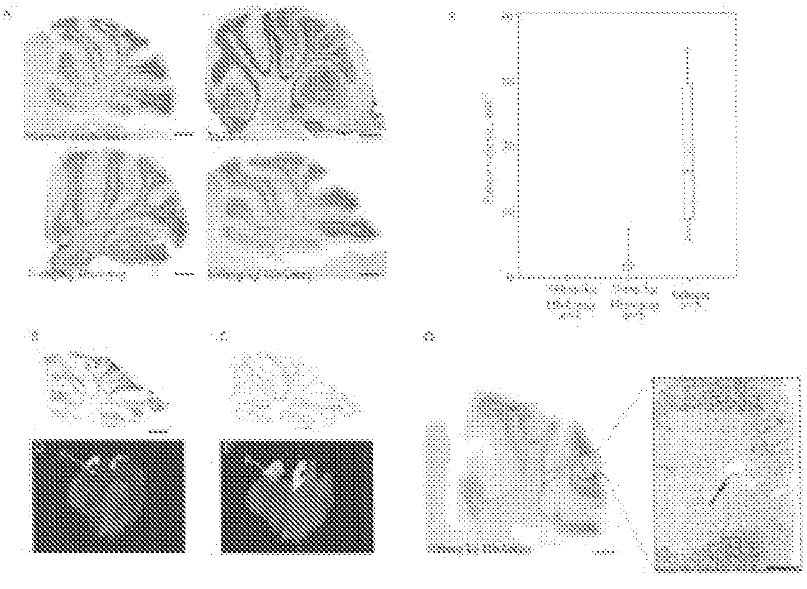

Figure 5. HhAntag given twice daily eliminates tumor mass in vivo

A: Sections of brain tissue from a wild-type mouse (normal cerebellum) and Ptc1$^{+/-}$ p53$^{-/-}$ mice treated twice daily with vehicle or HhAntag at 20 or 100 mg/kg are shown. Treatment was initiated at three weeks of age. Scale bar equals 500 μm.

B–E: Determination of tumor volumes using the Bioquant Nova 3D reconstruction program.

B: An example of a 5 μm sagittal section from an untreated Ptc1$^{+/-}$ p53$^{-/-}$ mouse, stained with cresyl violet. Scale bar equals 500 μm.

C: The outline of the IGL is indicated by the blue trace, and the regions of tumor are traced in the other colors. The different colors used for tracing the tumor area mark separate regions of tumor.

D and E: Sections were traced every 150 μm and assembled to create 3D images of the tumor (**D**) plus the IGL or the tumor alone (**E**). R: rostral direction.

F: Tumor volumes from vehicle-treated mice and from mice treated with HhAntag at 20 or 100 mg/kg are represented in a box plot. The Exact Kruskal-Wallis test suggests a clear statistical difference in the distribution of tumor volumes among the three groups ($p < 0.0001$), and the Exact Wilcoxon-Mann-Whitney test revealed a statistically significant dose-dependent decrease in tumor volume associated with HhAntag treatment ($p = 0.0159$).

G: Signs of tumor regression are present in some mice treated with 100 mg/kg of HhAntag twice daily for 14 days. The figure shows a low-power image in the left panel and a high-power view in the right panel of a lesion area (boxed in the low-power image). Infiltrating lymphocytes are indicated by a black arrow. Scale bar equals 500 μm for the low-power and 100 μm for the high-power image. All sections were stained with haematoxylin and eosin unless indicated otherwise.

free survival in Ptc1$^{+/-}$ p53$^{-/-}$ mice (the Exact Two-Sided p value for Log-Rank Test was 0.0001).

Discussion

In this study, an inhibitor of the Shh pathway, HhAntag, eliminated tumor in a mouse model of spontaneous medulloblastoma. HhAntag blocked the ability of Smo to increase *Gli1* expression in tumor cells and reduced tumor cell proliferation in vivo. At the highest dose used, there was no measurable tumor volume after 2 weeks of treatment. Remarkably, although tumor growth disrupts the structure of the cerebellum, cerebellar morphology was relatively normal in the treated mice. These findings provide strong encouragement for the development of small molecules that block Smo function to treat tumors in which the Shh pathway is activated. Our findings extend previous studies using cultured tumor cells and transplantable tumors in which cyclopamine was shown to inhibit growth of mouse and human tumor cells (Berman et al., 2002, 2003; Taipale et al., 2000; Thayer et al., 2003). However, we also demonstrate that the Shh pathway is downregulated in cultured mouse medulloblastoma cells and that inhibition of cell proliferation in culture using cyclopamine and HhAntag is not mediated by inhibition of Smo. In contrast, treatment of mice with HhAntag blocked tumor growth at the same concentration that inhibited Smo activity, as determined by the level of *Gli1* expression. This finding suggests that additional factor(s), present in the intracranial environment but missing in cell culture, are required to maintain the ability of tumor cells to respond to Shh. These results underscore the utility of genetically programmed mouse tumor models in which effects of targeted therapies can be determined in an appropriate in vivo context.

HhAntag penetrated the blood-brain barrier after oral delivery and efficiently suppressed tumor cell proliferation. Although apoptosis was increased in treated tumors, particularly at the highest dose used, this effect was modest. It is likely that the major mechanism of action of HhAntag is antiproliferative; how-

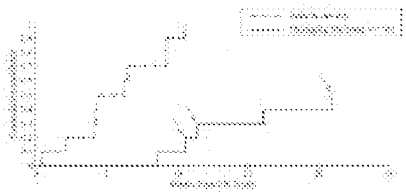

Figure 6. Treatment of Ptc1$^{+/-}$ p53$^{-/-}$ mice with HhAntag increases life expectancy

At 3 weeks of age, mice were arbitrarily assigned into two groups of ten. One cohort was treated once daily with 100 mg/kg of HhAntag, and the other with vehicle alone. The graph shows the cumulative incidence of euthanasia due to brain tumors in the presence of competing event: euthanasia due to other tumors. The Exact two-sided p value for the log-rank test was 0.0001, indicating a highly significant difference in brain tumor-free survival between HhAntag- and vehicle-treated mice. Due to the limited amount of drug available, after 30 days of treatment, drug was withdrawn from four of the eight mice still alive in the treatment group. Three of these mice subsequently developed medulloblastoma (the times of euthanasia are indicated by arrows).

ever, we cannot rule out the possibility that a peak of cell death occurs at an earlier time point during the treatment regime. The basal apoptotic rate in tumors is quite high, so inhibition of proliferation would result in loss of tumor volume by attrition. The loss of tumor volume was associated with macrophage infiltration and reactive gliosis but not necrosis. Future studies will address the temporal mechanism of action of HhAntag. During the course of our experiments, we did not detect any toxic side effects of the therapy. Even in prolonged studies, in which mice received 100 mg/kg of HhAntag every day for more than 50 days, there were no signs of deleterious effects or weight loss in the treated group.

Treatment of mice with HhAntag for 2 weeks at 100 mg/kg twice daily resulted in an absence of measurable tumor volume, suggesting that at this dose, the tumor was eradicated before drug resistance could develop. The high dose was crucial for the tumor response, as there was only a partial regression in the cohort of mice receiving 20 mg/kg. Furthermore, in the survival study, in which HhAntag was given at 100 mg/kg once a day, mice still succumbed to medulloblastoma, albeit with a significant delay compared to vehicle-treated mice. Thus, in considering the use of HhAntag for treating human disease, it will be important to use the appropriate dose to ensure complete suppression of Smo in tumor cells. Alternatively, it may be possible to identify additional inhibitors that display more favorable pharmacokinetics.

Although Gli1 is a target of Smo signaling, it is not yet known if Gli1 mediates the oncogenic activity of the Shh pathway. Recently, it was reported that a virus expressing Shh is capable of inducing medulloblastoma in mice lacking *Gli1* (Weiner et al., 2002). Since *Gli2* is functionally similar to *Gli1*, there may be redundancy and/or compensation among family members (Bai et al., 2002; Park et al., 2000). However, in addition to suppression of *Gli1*, we found that HhAntag also inhibited expression of several other genes in tumor cells in vivo, including *Gli2* (data not shown). It is possible that some or all of these genes contribute directly to oncogenesis. At present, it is not possible to distinguish between cause and effect: the changes in gene expression could be responsible for the effect on tumor growth, or the inhibition of tumor growth might have resulted in the changes in gene expression. We are now pursuing time course experiments and gene expression microarray studies to examine the differences in gene expression between treated and untreated mice. These studies will help to decipher the mode of action of HhAntag.

We were surprised to discover that, in contrast to *Gli1*, *Math1*, *Ptc2*, and *Sfrp1*, the level of *Ptc1* was not reduced after treatment with HhAntag. This was not expected, as *Ptc1* is induced in cell culture, including medulloblastoma cell cultures, by overexpression of *Gli1*. Previously, we reported that the wild-type *Ptc1* allele is expressed in medulloblastoma at a similar level to that observed in normal P7 and adult cerebellum (Wetmore et al., 2000, 2001). These findings contrasted with a report using reverse-transcriptase PCR analysis to indicate that expression of *Ptc1* is lost in medulloblastoma (Berman et al., 2002). These authors concluded that complete loss of *Ptc1* expression was necessary for medulloblastoma formation. However, our observation was confirmed by an independent study (Zurawel et al., 2000), and we have now extended these analyses using quantitative real-time PCR, in situ hybridization, Northern blotting (data not shown), and microarray analysis (Lee et al., 2003). Additionally, complete sequencing of the normal *Ptc1* allele, using cDNA from tumors and tumor cell lines, demonstrated that the remaining *Ptc1* allele is not mutated. Therefore, haploinsufficiency of *Ptc1* promotes medulloblastoma formation in *Ptc1*$^{+/-}$ and *Ptc1*$^{+/-}$*p53*$^{-/-}$ mice. In tumor cells, *Ptc1* is not subject to the usual positive feedback regulation, as *Ptc1* levels, unlike those of *Gli1*, are not downregulated following suppression of the Shh pathway by the Smo inhibitor HhAntag in vivo. This implies that there may be a defect in the autoregulatory loop of the Shh pathway in tumor cells in vivo.

The three-stage preclinical approach we devised to explore the effect of a small molecule inhibitor in our mouse model may have general application. In the first stage, we carried out a short-term study demonstrating that HhAntag inhibited its target, Smo, in tumor cells in vivo in a dose-dependent manner. The second stage involved longer-term exposure of mice to the compound followed by quantitative evaluation of the dose-dependent effects of HhAntag on tumor growth. The final stage, similar to human clinical trials, demonstrated that prolonged exposure improved the survival of mice with tumors. At each stage, the drug was delivered by oral gavage to mimic the potential dosing route in patients. To apply this approach, it is critical to develop a high-incidence, early-onset mouse model, consistent with the genetics of human tumors. It is likely that such models represent only one particular molecular subtype of the tumor, rather than the entire spectrum of the corresponding human disease. In our case, the activated Shh pathway in tumor cells allowed testing of the proof of principle that inhibition of Smo by a targeted therapy would block tumor growth in vivo. For this strategy to be successful, it is important to identify a reliable marker of pathway activity (*Gli1* expression level in our case) and to use compounds with good bioavailability in the target tissue. If these conditions can be met, the three-stage approach described here can provide information on dose-dependent activity, tumor pathology, potential side effects, and long-term survival.

Since HhAntag works by suppressing Smo activity, it should be effective against medulloblastomas in which upstream components of the Shh pathway have been activated by mutation. However, HhAntag may also have a broader application. Several mouse medulloblastoma models have been described in which *p53*, together with *PARP-1*, *Lig4*, *Rb*, or different cell cycle regulators have been mutated (Hesselager and Holland, 2003; Lee et al., 2003). These tumors exhibit similar gene expression profiles to *Ptc1*$^{+/-}$ medulloblastoma, including elevated *Gli1* levels, indicating that they may also be inhibited by HhAntag (Lee et al., 2003). Approximately 25% of sporadic human medulloblastoma harbor mutations in different components of the Shh pathway (Ellison et al., 2003). These tumors are excellent candidates for potential treatment with Smo inhibitors, and it is also possible that some of the remaining 75% of human medulloblastoma would respond to HhAntag. Inhibitors of the Shh pathway may also be efficacious against other tumors with an activated Shh pathway, such as most basal cell carcinoma (Aszterbaum et al., 1999), a subset of meningioma, breast carcinoma, and colon cancer (Xie et al., 1997). Suppression of Shh signaling could also be beneficial in tumors that do not harbor mutations in the Shh pathway, but in which Shh signaling is important for continuous tumor cell growth (Berman et al., 2003; Thayer et al., 2003).

The data presented here provide strong support for initiating human clinical trials with molecular inhibitors of the Shh pathway, such as the Smo suppressor HhAntag. The patient populations most likely to benefit from such intervention would include Gorlin syndrome patients and patients with sporadic medulloblastoma containing mutations in the Shh pathway. One important challenge in such trials would be to identify a marker that could serve as an indicator for dose-dependent suppression of Smo signaling. This could be achieved by monitoring changes in gene expression, for example, downregulation of Gli1. However, it may be necessary to utilize surrogate cells affected by the treatment, for example, epidermal cells in which the Shh pathway is active, or alternative tumor-specific markers such as secreted proteins that could be measured in blood or cerebral spinal fluid, to monitor the efficacy of pathway suppression.

Ultimately, it is likely that success in treating cancer will require the use of several compounds in concert that target distinct pathways important for tumor cell growth. Our mouse studies with HhAntag offer the hope that such precise, targeted molecular interventions could spare children from the devastating effects of surgery, toxic chemotherapy, and exposure to high doses of radiation.

Experimental procedures

Cell culture, luciferase, and cell growth assays

Mouse medulloblastoma cell cultures were established and characterized as previously described (Li et al., 2005). NIH3T3 and medulloblastoma cell cultures were maintained in DMEM (GIBCO-BRL/Invitrogen) under standard conditions. Cells were plated at 4 × 10^4 or 1 × 10^5 per well in a 24-well plate 24 hr before transfection. 0.25 μg of Gli-Firefly luciferase reporter plasmid (Sasaki et al., 1997) and 0.0125 μg of *Renilla* luciferase plasmid (pRL-SV40) mixed with Fugene 6 were applied to the cells (Roche Diagnostics). Cells were harvested 48 hr after transfection medium containing 0.5% FBS, ShhN, and HhAntag compound (Curis, Inc., Boston, MA) was applied to confluent cells. Luciferase assays were performed according to manufacturer's instructions (Dual Luciferase Assay Kit, Promega) and normalized for transfection efficiency using *Renilla* luciferase activity.

Cell proliferation was assayed in triplicates using Alamar blue reagent according to the manufacturer's directions. HhAntag was obtained from Curis. HhAntag is available from Genentech under a material transfer agreement, available at http://www.gene.com/gene/about/collaborations/contracts.jsp. HhAntag details are presented in the US patent WO 2003011219, available from the web site http://l2.espacenet.com/espacenet/viewer?PN=WO03011219.

Retroviral vectors and retroviral-mediated gene transfer

HA-tagged, full-length mouse *Gli1* cDNA was generated by PCR and subcloned into pCX4pur retroviral-mediated expression vector (GenBank accession #AB086386). Retroviral-mediated gene transfer was performed as described previously (Akagi et al., 2003). 72 hr after the infection, cells were harvested and used for the Northern blotting analyses.

Cerebellar granule neuron precursor culture and BrdU incorporation assay

Cerebellar granule neuron precursor cells were isolated from postnatal day 5 C57Bl/6 mice as previously described (Furuya et al., 1998) and plated at a density of 3 × 10^5 cells/cm^2 onto poly-D-lysine-coated plastic plates (BD Biosciences), with indicated amounts of ShhN and HhAntag. 10 μM BrdU was added 2 hr before cells were fixed with 0.5 M HCl, 70% EtOH. BrdU assays were performed using BrdU labeling and detection kit III (Roche Diagnostics) according to manufacturer's instructions.

Northern blot analysis

Total RNA was isolated from snap frozen tissue or cultured cells using the TriPure reagent (Roche Diagnostics), according to the manufacturer's instructions. 10 μg was separated on 1% agarose gel under denaturing conditions and transferred onto nylon membrane (Hybond-N⁺, Amersham Biosciences). After UV crosslinking, the membrane was hybridized using DNA probes for *Gli1* (NM_010296.1) nt866–2024, *Ptch1* (NM_008957.1) nt4181–4305 plus the 739 nucleotides of the 3′ UTR, *Ptch2* (NM_008958.1) nt682–3549, and *Smo* (NM_176996.3) nt2854–3682.

Mice

The mice used in this study were generated and maintained on a mixed C57Bl/6;129Sv background, as described previously (Wetmore et al., 2001). Mice were observed daily and euthanized according to NIH-approved institutional guidelines when showing signs of increased intracranial pressure or extracranial tumors were evident. Animals were housed in an American Association of Laboratory Animal Care-accredited facility and maintained in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at St. Jude Children's Research Hospital approved all procedures for animal use.

Real-time PCR analysis of gene expression

6-week-old *Ptc1*$^{+/-}$ *p53*$^{-/-}$ mice treated by oral gavage twice daily for 4 days, with HhAntag at 20 or 100 mg/kg or vehicle. 4 hr following the last dose, mice were euthanized and their tumor-bearing cerebella were snap frozen in liquid nitrogen. Total RNA was extracted using the TriPure reagent (Roche Diagnostics). 5 ng was analyzed using the real-time RT-PCR ABI Prism 7900 sequence detection system (Applied Biosystems). Levels of assayed genes were normalized to 18S rRNA abundance. The sequences of the amplification primers and Taqman probes are: *Gli1* primers 5′-GCTTGGATGAAGGACCTTGTG, 5′-GCTGATCCAGCCTAAGGTTCTC, probe 5′-CCGGACTCTCCACGCTTCGCC; *Sfrp1* primers 5′-TCCTCCATGCGACAACGA, 5′-TGATTTTCATCCTCAGTGCAAACT, probe 5′-TGAAGTGAGACGCCATGATTGAACATCTCTG; *Math1* primers 5′-ATGCACGGGCTGAACCA, 5′-TCGTTGTTGAAGGACGGGATA, probe 5′-CCTTCGACCAGCTGCGCGAAGG; *Ptc1* primers 5′-CAAGTGTCGTCCGGTTTGC, 5′-CTGTACTCCGAGTCGGAGGAA (detecting the wt *Ptc1* allele), probe 5′-CCTCCTGGTCACACGAACAATGGGTC, *Ptc2* primers 5′-TGGAGCCACCTTGGTACAAGA, 5′-GGCGCTCAGGAAAGCATGT, probe 5′-CTGGCGCTGACAGATGTGGTCCCT; and *Reln* primers 5′-CGCGATCGCCTGGTCTTTAT, 5′-TCTGTGACTGAGCAGGAGGCA, probe 5′-AAAAGGCCAGCACCCGTTAGGTGG.

Pharmacokinetics

Brains were snap frozen 4 hr after the last HhAntag dose, homogenized in PBS, and centrifuged. HhAntag was extracted from the supernatant using a Strata-X solid phase extraction column (Phenomenex 8B-S100-UBJ). The drug was eluted with methanol/acetonitrile/acetic acid (70:30:0.5:1:0), dried, reconstituted with 100 μl 70% acetonitrile water, and centrifuged, and supernatant was transferred into an autosampler vial for injection. Chromatographic separation was achieved on a Waters Xterra MSC$_{18}$ 3.5 μm, 30 × 2.1 mm column with an isocratic mobile phase of acetonitrile and water (50:50). The analytes were detected with a triple quadrupole mass spectrometer, and the ions were monitored in the multiple reaction monitoring (MRM) mode including m/z 481 (precursor ion) to m/z 150.9 (product ion) for HhAntag and m/z 378 (precursor ion) to m/z 244 (product ion) for HhAntag-358 (internal standard). The lower limit of quantitation of HhAntag was 5 ng/ml (signal/noise ≥ 5), and results from a 3-day validation study demonstrated acceptable within-day and between-day precision (CV% values ≤4.4% and ≤5.5%, respectively) and accuracy (range 104.4% to 108.0%).

In situ hybridization

Gene expression was evaluated by in situ hybridization as described previously (Rice et al., 1998). Probes for *Gli1*, *Ptch1*, and *Ptch2* are described above; *Sfrp1* (NM_013834.1) nt239–1205, *Math1* (NM_007500.2) nt832–1128, *Reln* (NM_011261.1) nt5269–5975. Probes were [α-^{33}P]UTP radiolabeled. Autoradiography proceeded for 1–10 days. Reference sections were stained with hematoxylin and eosin. Slides were photographed with a Nikon Coolscan E5 camera mounted onto a Zeiss Stemi 11 Stereo microscope.

Histological analyses

Mice were perfused intracardially with 4% paraformaldehyde and brains were removed and postfixed overnight at 4°C. Brains were dehydrated through a series of ethanols, delipidated in mixed xylenes, and embedded in Paraplast X-TRA (Fisher Scientific). Brains were sectioned at 5 μm in the sagittal plane and mounted on Superfrost + slides (Fisher Scientific). For histological analyses, sections were stained with hematoxylin and eosin using standard histological protocols. Slides were photographed using Axioplan Zeiss microscope (Zeiss) and RT color SPOT camera (Diagnostic Instruments).

Volumetric measurement of tumors

Stained sections taken every 100 μm were analyzed using the Bioquant Nova Advanced Image Analysis software. An outline of the remaining normal IGL and the tumor region was prepared for every section. This analysis was inclusive in that any abnormal tissue area was assumed to be tumor. Traces were aligned using the IGL outline as a reference and reconstructed into a three-dimensional image, and the volume for every tumor was calculated.

Immunohistochemistry and detection of apoptosis

Paraffin sections were processed for immunohistochemistry with antibody against Ki67 (Novocastra Laboratories) by deparaffinization and rehydration followed by antigen retrieval in 10 mM sodium citrate at 90°C. Slides were treated with 1% H_2O_2, blocked in 10% normal goat serum, and incubated with primary antibody (1:2000 dilution) overnight at room temperature. Immunoreactivity was detected using the ABC Elite kit (Vector Laboratories) and diaminobenzidine reagent set (Kirkegaard and Perry Laboratories), according to manufacturers' recommendations. Slides were cover-slipped and analyzed using an Olympus BX60 microscope. Cells undergoing apoptosis were detected using the DeadEnd Colorimetric TUNEL system (Promega), according to manufacturer's instructions. Proliferative and apoptotic indexes were determined by counting 500–1000 cells for each tumor.

Survival studies

A cohort of [illegible]-week-old $Ptc1^{+/-}$ $p53^{-/-}$ mice was treated with HhAntag at 100 mg/kg of body weight, or vehicle alone, by oral gavage, once daily. The mice were observed daily and euthanized if any sign of illness could be discerned. The presence of brain tumors was confirmed by gross examination of dissected brain.

Statistical considerations for the manuscript

StatXact 5 for Windows was used for all exact statistical analyses (Statistical Software for Exact Nonparametric Inference, CYTEL Software Corporation). The assumption that gene expression intensities were from Gaussian distributions was rejected by the exact Shapiro-Wilk's test (all p values < 0.05); thus, the traditional t test is not valid for analyzing these data. Exact Kruskal-Wallis tests were used to compare gene expression intensities, levels of proliferation and apoptosis, as well as tumor volumes between vehicle and treated mice. Cumulative incidence functions of mice euthanized due to brain tumors in the presence of competing events of euthanasia due to other tumors were estimated as discussed by Kalbfleisch and Prentice and compared using exact log-rank tests. All p values are for two-sided tests and those less than 0.05 were considered statistically significant without adjustment for multiple testing (Kalbfleisch and Prentice, 1980; Kruskal and Wallis, 1952; Shapiro and Wilk, 1965).

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Involvement of *Sonic hedgehog* in the Cell Growth of LK-2 Cells, Human Lung Squamous Carcinoma Cells

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Mutation of the *Patched* gene has been detected in human inherited basal cell nevus syndrome (BCNS) and sporadic basal cell carcinomas (BCC), suggesting a strong relation between a Sonic hedgehog Patched signal and cell proliferation. In the present study, we demonstrate that *Sonic hedgehog* is expressed in human lung squamous carcinoma (LK-2 and EBC-1) and some adenocarcinoma cell lines. The expression of *Sonic hedgehog* is also detected in the human lung squamous carcinoma tissues, but not in the normal lung tissue of the same patient. The N-terminal region of Sonic hedgehog stimulates the incorporation of BrdU into LK-2 cells and stimulates their cell growth, while anti-Shh-N inhibits their cell growth. These results suggest that a Sonic hedgehog signal is involved in the cell growth of LK-2 cells. © 1997 Academic Press

Sonic hedgehog (*Shh*), which is a vertebrate homologue of *hedgehog* (*hh*), a segment polarity gene, is specifically expressed in the notochord and floor plate in early developmental stages, and zone polarizing activity (ZPA) in the limb buds and epithelium of lung and digestive organs in later developmental stages (1-5). Very recently, a mutation was detected in human *Shh* in the patients of holoprosencephaly (6). Targeted gene disruption of *Shh* in the mouse shows abnormal development of the brain, spinal cord, axial skeleton, and the limbs (7). Thus *Shh* is involved in the various morphogeneses of vertebrates.

The *Patched* (*PTC*) gene, identified initially as the *Drosophila* segment polarity gene, encodes a transmembrane protein (8-12). Recently, it has been shown that PTC has an ability to bind with Shh, possibly as its receptor (13,14). Patched constitutively represses downstream targets and the hh signal relieves *ptc* repression, thereby inducing transcription of the downstream genes in *Drosophila* (15-17). The mouse mammary oncogene *Wnt1* and the human glioblastoma oncogene *GLI* are homologues of the *Drosophila* segment polarity genes, *wingless* and *Cubitus interruptus*, respectively, which are components of the Hh-PTC signal transduction pathway (18,19).

Recently, the relationship between gene pathways of Shh-PTC controlling embryonic development and those involved in regulating cell proliferation has become a focus of study. Shh (~45kDa) itself catalyzes the intramolecular cleavage that generates Shh-N (~20kDa) and Shh-C (~25kDa) (20). Shh-N, which is sufficient to induce the duplication of digits (21) and motor neurons (22,23), has an mitogenic effect on the presomitic mesoderm cells *in vitro* (24). Overexpression of Shh induces a mitogenic effect on mouse lung mesenchymal cells and skin keratinocytes *in vivo* (25,26).

The basal cell nevus syndrome (BCNS) is characterized by developmental abnormalities and by the postnatal occurrence of cancers, especially basal carcinomas (BCCs), the most common human cancer. Very recently, a heritable mutation in patients with BCNS and a somatic mutation in sporadic BCC were identified in human *PTC* gene (27,28). Human *PTC* is a strong candidate gene for BCNS. If so, it is very likely that *PTC* plays a role as a tumor suppresser. Moreover, a mutation is also found in *Shh* gene of the patients with BCNS (26). These results suggest that Shh-PTC pathway is closely associated with cell proliferation in several types of adult cells.

In the present study, we focus on the relationship between the Shh-PTC pathway and the cell growth of human lung squamous carcinoma (LK-2) cells. We show that Shh-N stimulates the incorporation of BrdU

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into LK-2 cells and their cell growth, while anti-Shh-N prevents the cell growth of LK-2 cells.

MATERIALS AND METHODS

Cell culture. Human lung cancer lines, squamous carcinoma cells (EBC-1 and LK-2), adenocarcinoma cells (RERF-LCOK, VMRC-LCD, ABC-1), lung small cell carcinoma cells (RERF-LCMA, SBC-1, and SBC-2), human squamous carcinoma cells of mouth (NA, HSC-2), human epidermoid carcinoma cells (A431) were obtained from the Japanese Cancer Research Bank (Tokyo Japan). Human neuroblastoma cells (NB-1, IMR-32), human leukemia cells (U937 and Jurkat cells), and human teratocarcinoma cells (NT-2) were obtained from ATCC. These cells were cultured in α-minimum essential medium containing 10% fetal calf serum. In experiments to examine the effect of Shh on the cell growth,

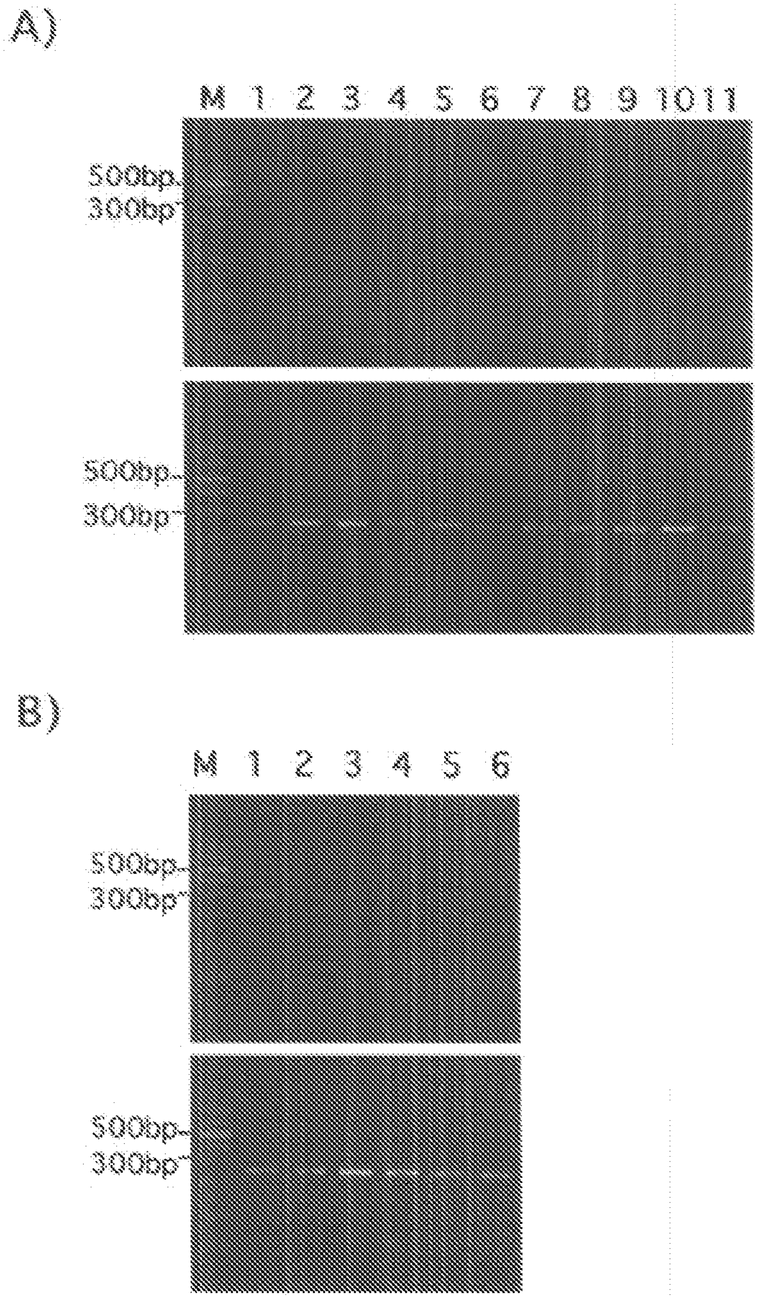

FIG. 1. RT-PCR of *Shh* and *PTC* of the human cancer cells. (A) Human lung cancer cells and squamous carcinoma cells. Lane 1, RERF-LCOK; lane 2, VMRC-LCD; lane 3, ABC-1; lane 4, EBC-1; lane 5, LK-2; lane 6, RERF-LCMA; lane 7, SBC-1; lane 8, SBC-2; lane 9, A431; lane 10, NA; lane 11, HSC-2. (B) Other human cancer cells, lane 1, LK-2; lane 2, NT-2; lane 3, NB-1; lane 4, IMR-32; lane 5, U937; lane 6, Jurkat cells. Upper panel is *Shh* and lower panel is *PTC*.

LK-2 cells were cultured with histag-Shh-N or anti-Shh-N at various concentration in the fetal calf serum free culture medium.

Incorporation of BrdU. Human lung carcinoma cells were inoculated at 1.1×10^{4} cells/cm² and incubated in the medium containing fetal calf serum for 8 h, followed with the incubation with serum free medium for 8 h, and histag-Shh-N was added. After incubation for 12 h, BrdU (3 μg/ml) was added in the culture medium and incubated for 1h. Then cells were washed with PBS and subjected to the detection of the incorporation of BrdU according to the manufacture protocol by immunostaining using anti-BrdU.

Preparation of His-tag fusion protein of Shh-N. Rat Shh-N was generated by polymerase chain reaction and subcloned into pET15b expression vector (Novagen). Restorations of reading frames were confirmed by DNA sequencing. The his-tag fusion protein of Shh-N was produced according to the manual. His-tag fusion protein was purified by Nickel-affinity column chromatography.

RT-PCR (reverse transcript-polymerase chain reaction). The expression of *Shh* and *PTC* in human cancer cells was examined by RT-PCR using 5'-CCAATTACAACCCCGACATC-3' and 5'-TCCTAGTACACCCAGTCGAA-3' for *Shh* and 5'-TATTACACCTTTGGACTGCTTCTGGGAA-3' and 5'-TTGAATTTTTGTTGGGGGCTGTGGCGGG-3' for *PTC*. Total RNA was prepared from human cancer cells according to the guanidium thiocyanate method (29). The total RNA (1 μg) was subjected to RT-PCR as described in the manual from Perkin-Elmer, 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 1 min, 30 cycles at 60°C for 2 min, and 1 cycle 60°C for 7 min. The PCR products were subjected to 3% Nusieve gel (Takara, Kyoto) electrophoresis.

Immunoblot analysis. The antiserum (anti-Shh-N serum) against RLAVEAGFDWVYYESC, synthetic peptide of Shh-N, was prepared (30). After anti-Shh-N serum was purified by peptide affinity column chromatography, reactivities of this antiserum against fusion protein of rat Shh-N were examined by immunoblot analysis. Recombinant His tagged proteins were solubilized with 6M urea from cells. Human Shh was extracted from LK-2 cells with 50 mM Tris-HCl (pH7.5) containing 150 mM NaCl and 1% Triton X-100. After centrifugation, aliquots (each containing 50 μg protein) were subjected into SDS polyacrylamide (12%) electrophoresis and immunoblot analysis. The proteins in the gels were electrophoretically transferred to nitrocellulose filter, and the filters were incubated overnight with anti-Shh-N at 4°C and then incubated with alkaliphosphatase-conjugated goat anti-rabbit immunoglobulin (Promega, Madison, WI). Immuno-reactivities were visualized by incubating the filter in the reaction buffer (100 mM Tris-HCl (pH9.5), 100 mM NaCl, 5 mM $MgCl_2$, 40 mM 5-bromo-4-chloro-3-indolylphosphate, and 80 mM nitro blue tetrazolium salt) at room temperature.

Immunostaining. The localization of Shh in lung squamous carcinoma cell lines, and the distribution of Shh in lung squamous tissues were examined by immunostaining using anti-Shh-N, which specifically react with lung epithelium of mouse embryos (30). After carcinoma cells and cancer tissue sections were fixed with 4% paraformaldehyde, they were incubated with anti-Shh-N antiserum at 4°C for 3 days, and the immunoreactivities on the cells and the each section were detected by a peroxidase-conjugated avidin-biotin kit (Vector Laboratories, CA).

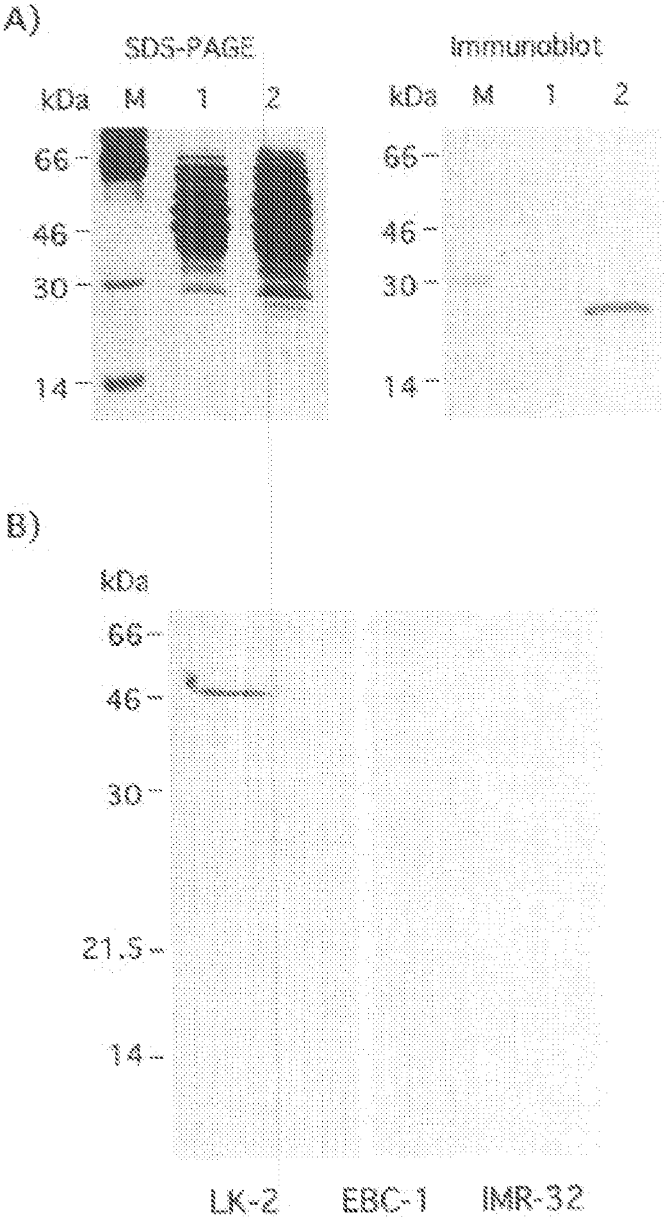

FIG. 2. Immunoblot analysis of Shh expressed in LK-2 cells by anti-Shh-N. (A) Reactivity of anti-Shh antiserum against histag-Shh-N fusion protein. Lane 1 correspond to cells transformed with pET15b vector alone, lane 2 to cells transformed with rat Shh-N conjugated with pET15b vector. (B) Immunoblot analysis of Shh expressed in human cancer cells. LK-2 cells (lane 1), EBC-1 cells (lane 2), and IMR-32 (lane 3).

RESULTS

Shh was expressed in the human lung squamous carcinoma (LK-2 and EBC-1) cells, weakly expressed in some of lung adenocarcinoma (RERF-LCOK, VMRC-LCD) and neuroblastoma cells (NB-1), but not in lung small cell carcinoma cells (RERF-LCMA, SBC-1, and SBC-2), leukemia cells (U937 and Jurkat cells), IMR-32 (neuroblastoma cells), NT-2 (teratocarcinoma) or mouth squamous carcinoma (HSC and NA) and A431 (epidermoid carcinoma cells) derived from ectoderm (Figure 1A). In contrast with *Shh*, *PTC* (250 bp) was detected by RT-PCR in all the cell lines examined (Figure 1B). The nucleotide sequences of PCR products of *Shh* and *PTC* of LK-2 cells was completely identical to those of *Shh* and *PTC* previously reported, respectively (10, 12, 31).

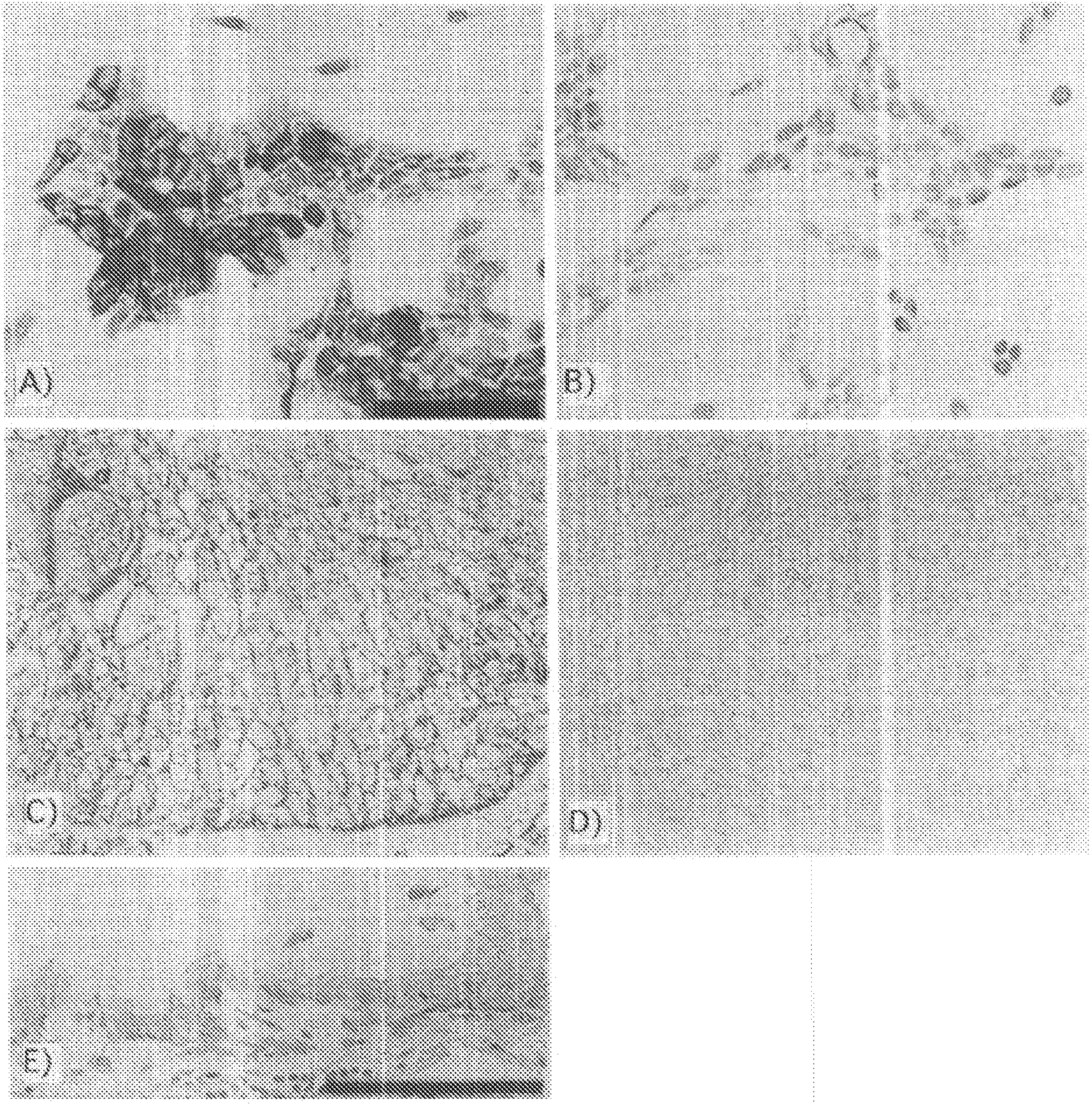

FIG. 3. Immunostaining of Shh of human lung squamous carcinoma cells and tissues. LK-2 cells (A and B), lung squamous carcinoma tissues from 58-year-old man (C and D) and normal lung tissues of the same patient with lung squamous carcinoma (E). Immunostaining with anti-Shh-N (A, C, and E) and immunostaining with normal rabbit serum (B and D). Bars indicate 100 μm.

Anti-Shh reacted with a 20 kDa band of histag-Shh-N fusion protein (Figure 2A). Human Shh (47 kDa) was detected in LK-2 cells and EBC-1 cells by immunoblot analysis using anti-Shh-N, but not detected in IMR-32 cells (Figure 2B). However, Shh-N (20 kDa), an endogenous cleavage fragment of Shh, was not detected in LK-2 cells and EBC-1 cells probably because of the small amount. Localization of Shh protein in LK-2 cells was examined by immunostaining using anti-Shh-N. Shh was mainly localized in the plasma membrane and partly in cytosol in LK-2 cells (Figure 3A), while Shh was negative in IMR-32 cells (unpublished observation). Shh was also positive in the three lung squamous carcinoma tissues (Figure 3C), while Shh was negative in the epithelium of normal lung tissues of the same patients (Figure 3E).

In the absence of fetal calf serum, Shh-N (1-1000 ng/ml) stimulated the incorporation of BrdU into LK-2 cells (Figure 4A). The stimulation of the BrdU-incorporation by Shh-N was shown in the lung squamous carcinoma cells, LK-2 and EBC cells, but not in the adenocarcinoma cells, RERF-LCOK and ABC-1 cells, and in the lung small cell carcinoma, RERF-LCMA and SBC-2 cells (Figure 4B).

Some population of LK-2 cells had an ability to maintain the cell growth in the absence of fetal calf serum autonomously. Shh-N (10 ng/ml) stimulated the cell growth of LK-2 cells, but not that of ABC-1 cells, 2-3 times more than unstimulated cells by 6-day incubation (Figure 5A and B). To examine the effect of Shh expressed in LK-2 cells on their cell growth, LK-2 cells were incubated in the presence or absence of anti-Shh-N. The cell growth of LK-2 cells was concentration dependently inhibited by anti-Shh-N, but not by normal rabbit immunoglobulin (Figure 5C). Thus LK-2 cells failed to grow in the presence of anti-Shh-N (100 ng/ml).

DISCUSSION

Shh is intensely expressed in the mouse lung epithelium during branching morphogenesis, and decreases during development (25,30,32). In adult mouse lung tissues, the expression of *Shh* is faintly detected. In contrast with *Shh*, *PTC* is expressed in the mesenchymal cells located near the epithelium of the distal tips of bronchial tubes, in which *Shh* is intensely expressed. Overexpression of *Shh* in lung epithelium has a mitogenic effect on the lung mesenchymal cells *in vivo* (23). Thus Shh secreted from lung epithelium has a growth effect on the mesenchymal cells by the Shh-PTC pathway by paracrine mechanisms.

As shown in Figure 3C, Shh was positive in the squamous carcinoma, but it was not detected in normal lung tissues of the same patient. Shh seems to disappear in the adult human lung epithelial cells during aging, but it seems to appear in the lung squamous carcinoma cells. Thus far, *Shh* has been shown to be positive in all human lung squamous carcinoma cells (LK-2 and EBC-1) (Figure 1) and human lung

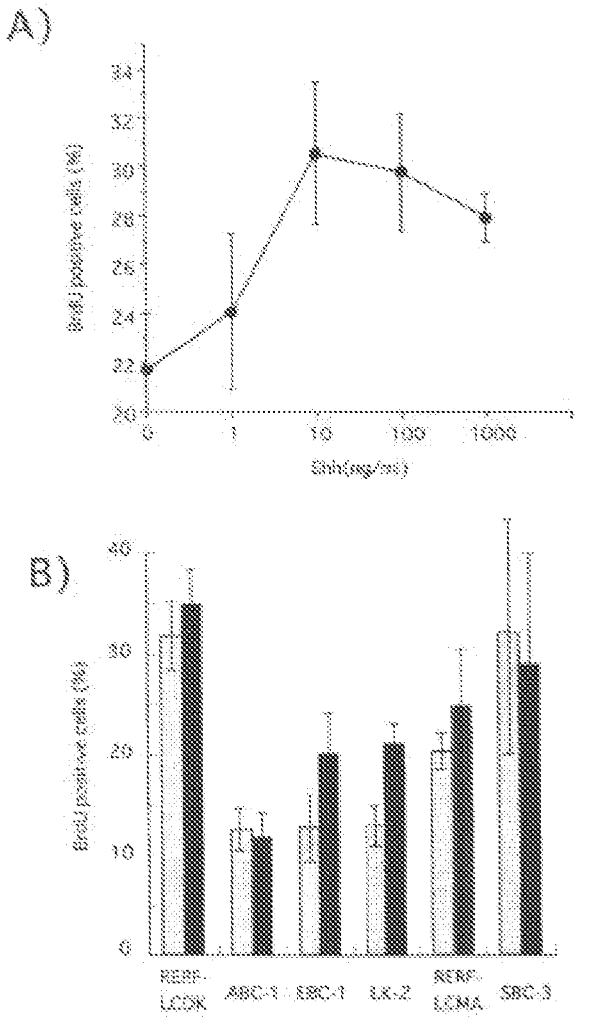

FIG. 4. Stimulation of BrdU-incorporation by Shh-N. (A) Dose-dependent incorporation of BrdU into LK-2 cells by Shh-N. (B) BrdU-incorporation into various lung carcinoma cells in the presence (black bars) or absence (gray bars) of Shh-N. Values are average of number of BrdU-positive cells of three experiments.

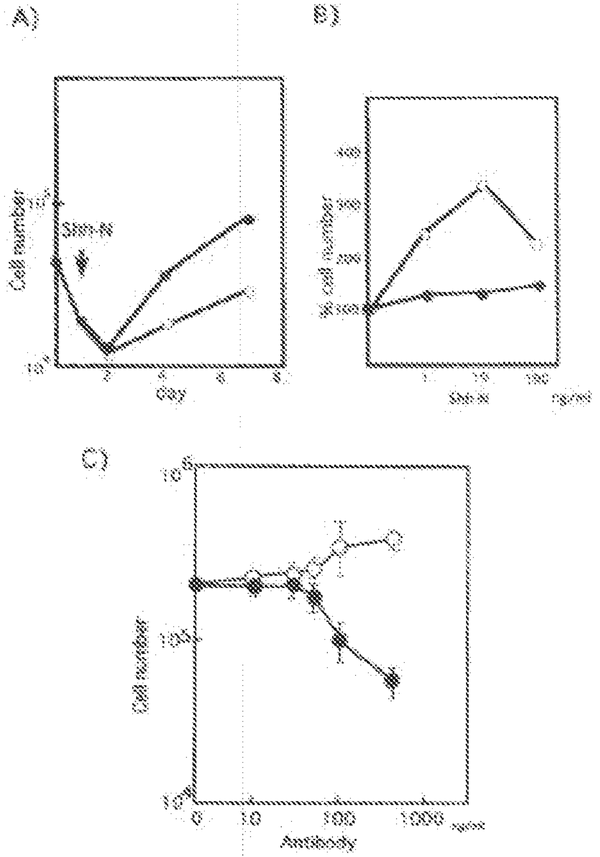

FIG. 5. Effect of Shh-N and anti-Shh-N on the cell growth of LK-2 cells. (A) The cell growth of LK-2 cells in the presence (closed circles) or absence (open circles) of Shh-N. Shh-N (10 ng/ml) was added at day one. (B) Dose-dependent effect of Shh-N on the cell growth of LK-2 cells (open circles) and ABC-1 cells (closed circles). (C) Effects of anti-Shh-N on the cell growth of LK-2 cells. LK-2 cells were incubated in the presence or absence of anti-Shh-N for 6 days. Cell growth of LK-2 cells in the presence of anti-Shh-N antibody (closed circles) or normal rabbit immunoglobulin (open circles).

squamous carcinoma tissues (n=3) we examined. In addition, other squamous carcinomas (T.T. and T.Tn.) derived from endoderm epithelial cells of the esophagus were Shh-positive (unpublished observation), while squamous carcinoma cells derived from ectoderm epithelial cells such as A431, NA, and HSC-2 were Shh-negative (Figure 1). Shh was weakly positive in the some of the lung adenocarcinoma, RERF-LCOK and VMRC-LCD (Figure 1), stomach adenocarcinoma (MKN-21), and colon adenocarcinoma (SW480) (unpublished observation). Thus the expression of *Shh* seems to be restricted to the squamous carcinoma and some of the adenocarcinoma derived from endoderm epithelial cells, which express Shh in the embryonic stage (5).

In contrast to *Shh*, *PTC* was expressed not only in the lung squamous carcinoma but also in all other cancer cells examined (Figure 1). However, if PTC is only a receptor for Shh, it is not clear why stimulation of BrdU-incorporation induced by Shh-N is restricted to lung squamous carcinoma, LK-2 and EBC-1, cells (Figure 4). One of the possibility is that there may be a mutation in *PTC* gene of lung squamous carcinoma cells as shown in the patient with BCNS or sporadic BCC (27,28). The other possibility is that the Shh-PTC signal is different in lung squamous carcinoma and other type of lung carcinoma cells. Recently, *Smoothened* (*Smo*) has been postulated to be one of the positive signaling units interacting with PTC (13,33). The expression of Smo or interaction between Smo and PTC may be different in lung squamous carcinoma and other type of lung carcinoma.

Shh-N stimulated the cell growth of LK-2 cells, while anti-Shh-N inhibited the cell growth of LK-2 cells (Figure 5). Thus Shh of LK-2 cells seems to stimulate their own cell growth through interaction with PTC by an autocrine mechanism. LK-2 cells are useful for the study on the Shh-PTC signal involved in the cell proliferation.

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confirm the role of p53 in the GSK3b-induced apoptosis of the melanoma cells.

Results and Conclusions Incubation of melanoma cells with increasing concentrations of DW1/2 (3 nmol/l–3 μmol/l) for 72 h led to concentration-dependent reduction of growth (IC50 ~300 nmol/l). Mechanistic studies showed that DW1/2 induced apoptosis in the melanoma cells via the intrinsic pathway and was associated with mitochondrial depolarization, caspase/PARP cleavage and induction of NOXA. Parallel experiments revealed little apoptosis induction in primary human skin melanocytes. Other structurally unrelated GSK3b inhibitors, such as LiCl and SB216763, were also able to induce apoptosis in the melanoma cells.

Using RNAi and pharmacological approaches, we demonstrated that p53 was required for the cytotoxic effects of the DW1/2. The DW1/2 compound was unable to induce cell death in melanoma cells with p53 mutations, further confirming the requirement for wild-type p53 in the cytotoxic effects of the GSK3b inhibition. The effect of p53 in DW1/2-induced cell death was not simply due to passive upregulation of protein expression as adenoviral-mediated overexpression of p53 was not able to induce apoptosis in the melanoma cells. Treatment of melanoma cells with DW1/2 was instead found to decrease levels of the p53 repressors, Mdm2 and Mdm4. The importance of Mdm2 downregulation in DW1/2 induced apoptosis was confirmed by treating the p53 wild-type cells with the p53/Mdm2 antagonist Nutlin-3. Taken together, our data provide a new strategy for the pharmacological activation of p53 in melanoma, which may be a viable approach for overcoming apoptotic resistance in melanoma, and offer new hope for rational melanoma therapy.

ABS-0182

Mechanism-based treatment of tumors

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A somatic stem cell resides normally in a niche where its self-renewal and survival is supported. Capability of a stem cell for asymmetric division is affected by a number of means, including the physical orientation of stem and support cells allowing only one orientation of the stem cell mitotic spindle. A growing body of evidence points out to the existence of tumorigenic stem cells having genetic/epigenetic changes that predispose to the growth of tissue (tumor) in which the proportion of cells with less differentiated features and greater resistance to apoptosis is increased in comparison with normal tissue.

We have described induction of the differentiation and apoptosis of tumor cells with efficiency and selectivity *in vivo* by use of an inhibitor of Hedgehog/Smoothened (Hh/Smo) signaling and the causation of rapid regression and disappearance of treated skin tumors (Tas S, Avci O. *Eur J Dermatol* 2004; 14:96–102). Hedgehog proteins appear to play an essential role in the maintenance of stem cells. Yet we have shown that normal tissue cells, including the putative stem cells in hair follicles, are well preserved under the conditions causing differentiation of practically all tumor cells. Another instructive finding is that the tumor cells that are induced to differentiate beyond a critical stage do not resume proliferation when the treatment is discontinued. It has been shown that, whereas the inhibition of tumor cell proliferation can be a consequence of the induction of tumor cell differentiation, a dosage of an Hh/Smo signaling inhibitor that causes inhibition of tumor cell proliferation does not necessarily induce tumor cell differentiation or apoptosis. Utilization of Hh/Smo signaling for several physiological functions in a patient may present a challenge in clinical practice. However, various considerations suggest that the anti-tumoral selectivity and efficacy that have been described (Tas S, Avci O, *Eur J Dermatol* 2004; 14:96–102) can be attained in general. Tumorigenic stem cells, unlike their normal counterparts, may be compromised in terms of proper orientation and adhesions with niche support cells and appear to rely more on the genetic/epigenetic changes of the Hh/Smo signaling components that cause inappropriately increased or constitutive Hh/Smo signaling. Further, the non-normal forcing of proliferation of tumor cells may result in the accumulation of genetic/epigenetic changes in these mutation-prone cells in their ability to respond to the normal tissue signals. When the signals that they rely upon are removed or altered, they may become more susceptible to apoptosis than normal cells. Evidence will be presented that melanocytic tumors may also commonly rely on Hh/Smo signaling for the maintenance of tumorigenic cells displaying stem cell features.

ABS-0183

Functional genomics of calcium channels in human melanoma cells

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Calcium (Ca^{2+})-signaling of human melanoma is in the focus of intensive research since the identification of the role of WNT-signaling in melanogenesis. Genomic and functional studies pointed to the important role of various Ca^{2+} channels in melanoma, but these data were contradictory. In the present study, we clearly demonstrate, in a number of different ways including microarray analysis, polymerase chain reaction (PCR), DNA sequencing and

A FUNDAMENTAL VULNERABILITY OF TUMOR CELLS

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Running Head : Vulnerability of tumor cells

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ABSTRACT :

Despite countermeasures, cellular proliferation increases the probability of mutation, crossing-over and gene-conversion events. Genetic stability is adversely affected also by alterations of chromatin structure associated with cellular differentiation. Having infrequently dividing stem cells that are kept in an undifferentiated state and maintaining soma through progenitor cells that differentiate while proliferating transiently is therefore an evolutionarily conserved strategy. As attested by the increased occurrence of tumors with aging, however, various means and strategies of improving genetic stability are incapable of preventing or fully repairing genetic damage. Unlike normal stem cells, tumor cells are independent of an anatomically defined niche as a consequence of the genetic and epigenetic changes they have acquired during tumorigenesis. Here I show that the very same essential changes that lead to the formation of a tumor render the tumor cells vulnerable and can be taken of advantage in treating tumors effectively.

Key Words : Apoptosis, Differentiation, Hedgehog, Niche, Tumorigenesis

Occurrence of an oxidizing environment on earth, within which the predecessors of present day multicellular organisms have arisen [1], and an error-prone gene replication machinery increase the mutation rate and genetic diversity upon which evolutionary selection may operate. Sexual reproduction, wherein pre-existing allele combinations are inevitably broken at each generation, contributes further to genetic diversity. Whereas a certain degree of errors in reproduction of genetic material may facilitate evolution, it is obvious that there is also a need for the limitation of such errors for preservation of advantageous genes and sequences in genome. Effective means of repair of DNA damage are known to be available to the germline during meiosis [2,3] and various means of improving genetic stability in somatic cells appear to have occurred during the evolution of longer lived organisms [4,5].

Organismal aging is associated with occurrence of increased frequencies of genetic and epigenetic changes in somatic cells [3, 6-9]. Among the various consequences of these changes, a marked age-dependent increase in tumor frequency is well documented [6-9]. Thus, the various strategies and means of even the most advanced of present day organisms are far from being capable of fully preventing and/or repairing the damage to genetic material in somatic cells. Here I show that the essential changes leading to the formation of a tumor can be taken of advantage in treating tumors effectively.

Utilization Of Infrequently Dividing Stem Cells Capable Of Giving Rise To Transiently Proliferating Differentiation-Committed Progeny Is A General Strategy In Maintenance Of Soma

That an error-prone gene replication and maintenance machinery may provide advantages in adaptation to changing environmental conditions but is disadvantageous in the maintenance and functioning of soma appears to have been dealt in the long lived organisms by the evolution of better damage prevention and repair mechanisms [4,5]. In addition, existence of infrequently dividing stem cells that are capable of giving rise upon demand to the differentiation-committed, transiently proliferating progeny (i.e., the progenitor cells and their descendents) would serve to decrease probability of tumorigenesis. Although various mechanisms that delay or inhibit proliferation of the cells with DNA damage act against fixation of damage as mutation, the mutation, crossing-over and gene-conversion events certainly take place during mitosis [10]. Available estimates of the frequencies of such events show that genetic changes may accumulate in the more mature cells to functionally relevant levels, particularly in those cell lineages requiring large numbers of mitoses from the stem to mature cell [10,11]. A general strategy evolved at the face of this situation appears to be limitation of the proliferation potential of the differentiation-committed daughter cell of an asymmetrically dividing stem cell (see below). Elimination by programmed death is an additional strategy that is particularly evident in those cell types that are subjected to increased damaging of DNA under physiological or pathological circumstances (e.g. granulocytes).

First critical events towards limitation of proliferation potential may take place already in the differentiation-committed daughter cell of a stem cell. Thus, it is well documented in embryonal stem cells that induction of differentiation is accompanied by a rapid repression of telomerase reverse transcriptase (TERT) [12,13]. This would allow proliferation of the differentiation-committed cells but would place normally an ultimate limit on proliferation. Besides an infrequent division, stem cells are found to have additional advantages in protection of genomic integrity. Thus, stem cells have in general not only better protection against the occurrence of DNA damage (e.g. more effective prevention of oxidative damage) [13] but also more efficient repair of damage in comparison to their differentiated progeny [13,14]. Alterations of chromatin structure that are associated with differentiation and the loss of telomerase activity appear to place constraints on DNA repair processes [15].

Stem cells in an organism are found typically in niches that support their maintenance and that also contribute to the regulation of stem cell proliferation and differentiation. In Drosophila, the germ line stem cells exist adjacent to the niche support cells and make adherens junctions with some of them [16-18]. High concentrations of the Drosophila homologue of APC (adenomatous polyposis coli) protein exist in these adherens junctions along with E-Cadherin and β-Catenin and may help formation of mitotic spindle in stem cell in only one orientation [17,18]. Thus, orientation of mitotic spindle orthogonally to the supporting niche cell provides a mechanism whereby one of the daughter cells is expelled out of the niche. The daughter cell expelled from the niche is found to be committed to differentiation while the daughter remaining in the niche can be maintained as a stem cell [17,18]. Studies in higher organisms are as yet limited but available evidence is consistent with the existence of similar mechanisms in them as well [18].

Careful experiments with another stem cell type in Drosophila have provided further insight into the regulation of differentiation of a stem cell [19]. The neuroblast, derived from the neural epithelium, displays stem cell features and divides asymmetrically to give rise to a differentiation-committed daughter cell (the ganglion mother cell-1-1A, GMC-1-1A) and another neuroblast (neuroblast-1-1A) [19]. In the asymmetrically dividing neuroblast, the cell fate determining protein Prospero is found localized to the basal side and the mitotic spindle is oriented in the basal to apical orientation. In this case, only the differentiation-committed daughter cell (GMC) receives the Prospero [19]. A homeo-domain containing protein of drosophila, Eve, is dependent on Prospero for expression and is expressed in the GMC lineage, but only after the GMC-1-1A proliferates [19]. By use of a specific inhibitor of DNA replication (aphidicolin), it was demonstrated that expression of Eve is absolutely dependent on the proliferation of GMC-1-1A and that Eve is expressed only after the cell progresses through S phase [19]. This progression through S phase is required for the chromatin of the gene for Eve to be remodeled so as to enable expression of Eve [19]. An absolute dependence of cellular differentiation on proliferation is found with several other cell types as well [20-22]. Greater accessibility of DNA in chromatin during replication and the opportunity provided for chromatin remodeling appear to be fundamental reasons for the proliferation-dependent differentiation [19, 21-23]. Eukaryotic genome and chromatin structure (which preceded the evolution of differentiated multicellularity) provide means of stable epigenetic inheritance of gene expression patterns, including through the differential accessibility of promoter and enhancer sequences [23]. Cellular proliferation thus provides an opportunity for differentiation utilized commonly by progenitor cells [20,21].

Features and mechanisms of action of signaling molecules acting on stem cells and on their differentiating progeny support again the interpretation that stem cell proliferation is sought to be kept to a necessary minimum and that the cells needed for maintenance of soma are provided principally through the proliferation of progenitor cells and of their descendants (which may differentiate while proliferating). A relatively small number of signaling molecules are found to act similarly on stem cells and on their immediate progeny in different organisms. As noted below, the pathways of signal transduction by these molecules can interact (a network may be a more proper term) and they may act jointly in the generation of a particular phenotype. Thus the responses to some of these signals can be context-dependent. Selected signals and their interactions that are informative about the functioning of stem cells are considered herein.

Wnt/β-Catenin Signaling

Binding of a Wnt family protein to its receptor complex on a cell commonly initiates a series of events culminating in the translocation of β-catenin to the cell nucleus to bind with TCF/LEF family transcription factors to the target genes to affect transcription [24]. The canonical Wnt/β-catenin signaling appears to function primarily to support proliferation of the differentiation-committed progeny of stem cells, i.e. the progenitor cells and their descendents. In hydra, which are among the earliest multicellular organisms, Wnt/β-catenin signaling is employed in generation of increased numbers of differentiated cells from the stem cell pool [25]. In higher organisms Wnt/β-catenin signaling is employed similarly in the proliferation of progenitor cells, which typically differentiate along a lineage while proliferating [26-28]. In hair follicles, Wnt/β-catenin signaling is found to upregulate expressions of a large number of proliferation promoting genes (15 or more) in the cells exiting the stem cell compartment [27]. Responses to Wnt/β-catenin signaling can be modulated powerfully by co-existing signals and previous exposures of cell [29]. In intestines, the enterocytes at the lumenal end are terminally differentiated under normal circumstances and have a relatively short lifespan before being shed or removed by apoptosis to give way to the upcoming cells from lower regions of crypt [24]. The crypt bases host stem cells and in situ analyses show that highest Wnt/β-catenin signaling activity exists near the crypt bases along with proliferative activity [24,30]. Reciprocal signaling between the epithelial and mesenchymal cells appear to be utilized in downregulation of Wnt/β-catenin signaling as well as of proliferation in the enterocytes towards lumenal side [24,30].

Role of Wnt/β-catenin signaling in stem cells has been relatively difficult to elucidate due in part to the context-dependence of cellular responses to said signaling and to the existence of numerous Wnt family proteins, even in Drosophila. However, available data is consistent with the interpretation that canonical Wnt/β-catenin signaling, if anything, is insufficient for the maintenance of stem cells and that the upregulation of said signaling serves primarily for the proliferation of differentiation-committed progeny of stem cells (cf, ref. 31). Wnt/β-catenin signaling can be a direct target of the Hedgehog/Smoothened (Hh/Smo) signaling. Genetic and functional investigations in Drosophila demonstrate that Ci, which mediates the intranuclear actions of Hh/Smo signaling, causes upregulation of Wg expression when the target cell receives Hh [32,33]. Furthermore, loss of Hh/Smo signaling causes loss of Wg-dependent phenotypes [33]. Hh/Smo signaling may modulate the Wnt/β-catenin signaling dependent phenotypes also indirectly, e.g. through TGF-β/Bmp signaling [34].

Hedgehog/Smoothened Signaling

The gene for Hedgehog (Hh) was identified first in Drosophila through its effects on embryo polarity. Involvement of Hh in embryo patterning as a direct inducer of differentiation of various cell types in a concentration-dependent manner and also as inducer of expression of diffusable intermediaries is well established [35]. In Drosophila, signaling by Hh is initiated by the binding of Hh to a membrane protein, Patched, which exerts an inhibitory action on another membrane protein, Smoothened (Smo), when unliganded [35]. Binding of Hh to Patched relieves its inhibitory action on Smo, which then initiates a series of events that result in the translocation of a bifunctional protein, Ci, to the cell nucleus [35]. In the absence of signaling by Hh, Ci is processed proteolytically to generate a transcriptional repressor [35]. Signaling by Hh causes inhibition of phosphorylation of specific amino acids on Ci to inhibit proteolysis and, when the amount of Hh reaching to the cell is sufficiently high, freeing of the full length Ci from the proteins that keep it out of nucleus [35]. Full length Ci acts in the cell nucleus as a transcriptional activator of the target genes having a consensus sequence for Ci binding [35]. General features of this signaling are found conserved in most multicellular organisms, although the signaling gets more complex in higher organisms [35]. For example, in human three different Hh proteins, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) exist and have distinct functions [35]. Similarly, three different homologues of Ci have been identified in vertebrates (Gli1, Gli2 and Gli3) along with additional regulatory proteins that exist upstream of Gli proteins [35]. The additional means of regulation of Hh/Smo signaling would serve the needs of more complex higher organisms. Hh /Smo signaling plays in this respect essential roles in the formation and functioning of central nervous system and Shh appears to have experienced intensified evolution in primates, particularly in the lineage leading to humans [36].

Hh/Smo signaling has features that facilitates its amplification as well as extinguishment depending on the needs of organism. For example, at least Gli1 and Gli3 are themselves transcriptional targets of Hh/Smo signaling and provide potential of signal amplification [35]. In addition, the gene for Patched also has a functional Gli binding sequence in its promoter region and the arrival of a Hh protein onto a target cell commonly causes increased expression of Patched [35,37]. The upregulation of Patched expression by Hh/Smo signaling creates a situation that is highly suited for the patterned differentiation of cells during development and in postnatal life. It makes Hh/Smo signaling suited as a master regulator in that regard. Patched is known to bind the extracellularly secreted Hh protein and to limit its domain of diffusion and, when a ligand is lacking, Patched inhibits Smo potently [35]. Thus, the initiation of signaling by a Hh protein initiates, at the same time, the events that can supply a protein that is capable of preventing the unchecked expansion of signaling both spatially (in the tissue) and temporally (in the cell). This is a feature that is particularly relevant since Hh proteins can impart different fates on cells depending on their concentration and also depending on the duration of exposure of cell to a Hh protein [38]. There is accumulating evidence that the diffusion range of secreted Hh proteins is regulated by additional means as well, e.g. by extracellular proteoglycans, the synthesis and deposition of which are affected by Hh/Smo signaling [39,40].

In both Drosophila and vertebrates, reciprocal signaling in tissues between the Hh producing cells and target cells is an additional means of regulating Hh/expression and also the tissue responses to the effects of downstream targets of Hh/Smo signaling. For example, in both the developing limb bud and tooth germ, mesenchymal cells near the Shh producing ectodermal/epithelial cells show Hh/Smo signaling activity and these mesenchymal cells express Bmp4 as a result [41]. Crucially, the effect of Bmp4 on the Shh producing cells is concentration dependent. Both insufficient Bmp4 activity and too high Bmp4 concentrations cause loss or inhibition of Shh expression in both the limb bud and tooth germ and both in vitro and in vivo [41]. Thus, there is a feedback regulation from the nearby cells on Shh producing cells. Diffusion of too high amounts of a Hh protein too far from the Hh producing cells is therefore unlikely to persist under normal circumstances.

An essential requirement has been described for Hh/Smo signaling in proliferation of somatic stem cells in Drosophila ovary [42]. Similarly, placement of bone marrow cells on top of feeder cells engineered to express a Hh protein caused marked expansion of the cells having features of hematopoietic stem cells [43]. In Drosophila ovary, Hh producing cells are found adjacent to the stem cells, either in touch with them or localized a few cells away at a distance that is clearly within the diffusion range of Hh [44]. Thus, at least in case of the well defined stem cell niches in Drosophila, the niche cells appear to provide stem cells with high concentrations of Hh. Despite the showing that proliferation of a somatic stem cell in its niche normally depends on provision of Hh signals [42], it is not known what factor(s) maintain the expression and secretion of Hh proteins by niche cells. However, a number of morphological, functional and genetic investigations in Drosophila point together to an attractive candidate. Engrailed (En) is a protein that is expressed in developing Drosophila embryo in a pattern overlapping and nearly identical with that of Hh [45] and is expressed in the adult Drosophila ovary in the same cells that also express Hh and create a niche for stem cells [44]. Whereas ectopic expression of either En or Hh in adult Drosophila ovary was unable to induce expression of the other [44], these findings do not exclude an interaction of en and hh in the niche cells towards the maintenance of high level expression and secretion of Hh by these cells. For example, chromatin structure of the en and hh genes in the cells of adult ovary, other than in the niche cells, may render them unresponsive to the ectopically provided signal. Ectopic expression of Hh in the imaginal disc of developing Drosophila causes, in this respect, ectopic expression of En in the same cells and, furthermore, this En goes on to activate expression of the endogenous hh gene [46]. Indeed, structural and functional analyses of the en and hh genes and of their products along with their expression patterns strongly suggest that the product of en may be a key player not only in the maintenance of high level Hh expression by niche cells but also in preventing inappropriate responses to the high level Hh by these cells themselves. Thus, whereas En has a large repressor domain in accord with its known functioning as a repressor of several target genes, it can clearly function also as a transcriptional activator in the presence of suitable co-activators [47]. The genes activated by En include the en itself and hh [47]. Furthermore, En is found to act as a repressor of the genes for Patched and Ci [47]. These features appear to make En almost perfectly suited for the dual task of maintenance of high level Hh expression by the niche cells and, at the same time, prevention of inappropriate responses by these cells to Hh, e.g. in the form of proliferation through autocrine stimulation.

Mechanism-Based Treatment Of Tumors

Tumors are tissue growths in which the proportion of cells with less differentiated features and increased resistance to certain apoptotic stimuli is increased in comparison to normal tissue. Tumor cells are typically heterogenous in their appearance as well as in their associations with other tissue components but within tumors there are often areas where tumor cells form structures resembling those in the normal tissue of origin [48]. Tumor cells displaying features of differentiation along a lineage are detectable in most tumors and both benign and malignant tumors can have terminally differentiated tumor cells (e.g. keratin pearls in squamous cell carcinoma) [48]. Thus, occasional cells in a population of tumor cells (which in general are genetically unstable) can be caused to differentiate by suitable external signals and cell-intrinsic features but this is typically inhibited in many other cells in a tumor. Studies in which clones of individual tumor cells are examined with and without prior exposure to an inducer of tumor cell differentiation together with the examinations of same cells in the presence and absence of a specific inhibitor of cellular proliferation show that the tumor cells that are committed to differentiation continue to proliferate for several cell cycles and that these tumor cell proliferations are absolutely necessary for the differentiation of tumor cells [49,50]. The tumor cells that are induced to differentiate are found to cease proliferation later on when they become terminally differentiated [49,50]. These findings, along with the considerations mentioned earlier concerning the basic strategies in multicellular organisms in maintenance of soma question rationality of the attempts at treatment of tumors by aiming inhibition of tumor cell proliferation *per se.* Not only that cessation of cellular proliferation can occur as a consequence of differentiation along a lineage (and this is an evolutionarily conserved strategy in multicellular organisms) but differentiation is often found depended on cellular proliferation, probably due in part to the opportunity provided for chromatin remodeling by replication.

Probability of tumorigenesis increases with organismal aging in association with the age-related increase in frequencies of mutations (nucleotide sequence changes) and epigenetic changes in somatic cells [6,9]. The genetic/epigenetic changes associated with tumorigenesis are found to promote tumor growth commonly by causing activation of a proliferation signal, an arrest along a differentiation path and/or by inhibition of apoptosis [51]. Typically, the probability of occurrence of such genetic/epigenetic changes is increased by other genetic/epigenetic changes that cause decrease of genetic stability in tumor cells [52,53]. Such genetic instability and the selective pressure in normal tissues may lead to the evolution of tumor cells that are even less responsive to the normal tissue signals at later stages of tumorigenesis. Tumors appear to develop due to the arisal of cells that are resisting to the differentiation signals from normal tissue and that are proliferating even in the absence of a stimulatory signal from therein because of the genetic and epigenetic changes they have acquired.

Whereas stem cell niches have in general been characterized incompletely, available evidence is consistent with an essential role of Hh/Smo signaling in the maintenance of stem cells in their niches. Drosophila ovary has perhaps the best characterized niches and, as already noted, special arrangements appear to exist therein to provide stem cells with high concentrations of Hh. The differentiation committed daughter cell of an asymmetrically dividing stem cell and its immediate progeny, however, are also within a range of diffusion of Hh from the Hh producing cells. Thus, the proliferating and simultaneously differentiating progenitor cells and their progeny are likely to be exposed to a gradient of Hh concentration, highest near the stem cell and lower further away from the niche. Defining features of stem cells include an infrequent division and being kept in an undifferentiated state. How the decisions concerning stem cell division are made is not clear but the translocation of β-catenin to the cell nucleus and the activation of Wnt/β-catenin signaling seems to be a plausible signal. As for the maintenance of an undifferentiated state, Hh/Smo signaling appears well suited. Hh at relatively high concentrations in and immediately around the niche may maintain the stem cell and may prevent premature differentiation of progenitor cells therein, whereas the relatively lower Hh concentrations further away may coincide with the appearance of differentiated progeny. In this respect, we have found that antibodies (BerEP4 and C8/144B) that label the hair follicle outer root sheath, occasional epidermal basal layer cells and the undifferentiated basal cell carcinoma (BCC) cells show a clear gradient pattern of labeling of the BCC nodules that are exposed to a specific inhibitor of Hh/Smo signaling (cyclopamine) by diffusion from one side [40]. In other studies, these antibodies marked the label retaining cells (presumptive stem cells) and undifferentiated progenitor cells in the hair follicle outer root sheath [54] and the BCC cells were found to display features of stem cells [55].

Constitutively increased Hh/Smo signaling is found in all BCC's as a result of the loss-of-function mutations of patched and/or gain-of-function mutations of smo [56, 57]. Similarly, constitutive or inappropriately increased Hh/Smo signaling activity is found in a wide variety of human tumors which apparently do so through similar means and/or through expression of a Hh protein causing autocrine/paracrine stimulation [58-64]. Tumor cells in all these tumors thus appear to have become independent of an anatomically defined niche by making their own Hh protein and/or as a result of constitutive ligand-free Hh/Smo signaling.

Dependence of tumor cells for inhibition of differentiation and for continued proliferation on the genetic and epigenetic changes that they have acquired, instead of a stem cell niche, provides an opportunity for treatment of tumors. We have shown that a specific inhibitor of Hh/Smo signaling (cyclopamine) can be used for inducing differentiation and apoptosis of tumor cells in vivo with efficiency and selectivity, causing rapid regression and disappearance of tumors and no clinically detectable adverse effects in patients [40,65]. In these investigations, an insufficient dosing of cyclopamine was found to leave behind undifferentiated tumor cells that could proliferate in the absence of treatment. A further instructive finding has been that the tumor cells that are induced to differentiate beyond a critical stage do not resume proliferation when treatment is discontinued [40]. Thus, cessation of tumor cell proliferation can indeed be obtained *in vivo* as a consequence of differentiation of tumor cells. With a sufficient dosing of cyclopamine, all tumor cells are caused to cease expressions of the proteins found in the putative epidermal/hair follicle stem cells [40,65]. Instead they upregulate expressions of the antigens known to be upregulated in the differentiating progeny of normal progenitor cells [40,65]. However, with these sufficient doses of cyclopamine, only a few residual tumor cells are found to express the antigens expressed normally by the more differentiated upper layer epidermal cells (detected e.g. by the Ulex Europaeus lectin type 1), evidently because most of the tumor cells that have started to express early markers of differentiation die by apoptosis, which is massive at sufficient doses [40,65]. Indeed, when the tumor regions exposed to relatively lower concentrations of cyclopamine are followed up for a few days without further treatment and then excised, frequencies of the cells displaying features of more differentiated progeny (including the keratin pearls) are increased [40]. Thus, it appears that the tumor cells that are induced to differentiate are rendered unable to survive when a sufficient dosage of cyclopamine is administered. Crucially, the normal tissue cells, including the putative normal stem cells, are well preserved at doses of cyclopamine causing rapid regression and disappearance of tumors [40,65]. Further, former treatment sites of skin onto which cyclopamine had been applied in similar or identical doses and were left unexcised show healthy looking normal skin and hair in long term follow up, consistent with the functional preservation of normal stem cells [40,66].

Whereas an absolute dependence of normal stem cells on Hh/Smo signaling [42] may be against the use of an inhibitor of said signaling for treatment of tumors, our investigations have shown possibility of the use of cyclopamine for causing disappearance of tumors while sparing normal stem cells. There is good reason for attainment of similar efficacy and selectivity in general by adjusting the dosage to a level that is sufficient to induce tumor cell differentiation and apoptosis. The genetic and epigenetic changes that render tumor cells independent of a normal stem cell niche and the selection of these genetically unstable cells under circumstances of upregulated Hh/Smo signaling may render them highly vulnerable when the critical signaling that they rely upon is inhibited or eliminated for a sufficient duration by a suitable dosing of an inhibitor of Hh/Smo signaling. The same dosing is likely to cause some degree of inhibition of Hh/Smo signaling in the normal stem cells as well but these cells are supplied continuously with high concentrations of a Hh protein in their niches. The tumor cells residing in an environment away from a niche, on the other hand, would be rendered devoid of Hh/Smo signaling by a sufficient dosage of an inhibitor of said signaling. Therefore, a dosage of an inhibitor of Hh/Smo signaling that induces differentiation and apoptosis of the tumor cells may leave normal stem cells unharmed and they would recover from the effects of a possible transient inhibition of Hh/Smo signaling, owing to the continuous high level supply of a Hh protein by the niche support cells.

Published studies wherein an inhibitor of Hh/Smo signaling is administered to a tumor bearing subject are limited at present. In a study with mice having medulloblastoma, Sanchez and Ruiz i Altaba reported that intraperitoneally administered cyclopamine caused inhibition of Hh/Smo signaling in the tumor cells residing in cerebellum and provided marked inhibition of tumor cell proliferation and of tumor growth along with improved cerebellar functions [67]. They reported further that a dosing or concentration of cyclopamine that caused significant inhibition of tumor cell proliferation in vivo or in vitro did not cause a significant change in tumor cell viability and that induction of tumor cell apoptosis required higher doses or longer periods of treatment [67]. These findings accord with ours. Administration of an inhibitor of Hh/Smo signaling beyond that sufficient for inducing tumor cell differentiation and apoptosis, on the other hand, would also be inadvisable. Although the amount of cyclopamine to be administered for a tumor could be determined without difficulty in our investigations (which is consistent with an advantageous efficacy/toxicity profile), employment of Hh/Smo signaling for a number of physiological functions in an adult [68,69] cautions against administration beyond what is needed.

Hh/Smo signaling can alter expressions of a large number of genes and be affected by numerous gene products [70]. Changes of expression in which of the potential target genes of Hh/Smo signaling are essential for the formation of a particular tumor or tumors in general is unknown at present. However, available evidence suggests that Hh/Smo signaling may indeed provide, through products of its direct target genes and through secondary intermediaries, the critical needs of a tumor for survival and growth. For example, Hh/Smo signaling has been well known to be employed for vasculogenesis and angiogenesis [71-73]. Similarly, expressions of a number of gene products known to be used for the undifferentiated maintenance of stem cells are upregulated by Hh/Smo signaling [74-76]. Modulators of the functions of some of these downstream targets and of upstream regulators of Hh/Smo signaling may also find beneficial use in treatment of tumors in certain settings. However, the utilization profile of a potential target for various functions in an organism generally limits the possibility of a therapeutic window. Intervention directly with Hh/Smo signaling with an aim to induce differentiation and apoptosis of tumor cells, on the other hand, appears to hold distinct advantages in treatment of tumors.

Conflict of Interest Statement : I have patents and patent applications in subject matters relating to those in this article (issued on the basis of international applications PCT/TR01/00027 and PCT/TR03/00017); I declare otherwise no relationship that might be considered to pose a conflict of interest.

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